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A STUDY OF CORYNEFORM BACTERIA FROM MILK AND OTHER SOURCES WITH PARTICULAR REFERENCE TO *CORYNEBACTERIUM BOVIS*

This thesis is submitted to the University of Glasgow for the degree of Doctor of Philosophy

by

Wilkie Francis Harrigan, B.Sc.

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CONTENTS

GENERAL INTRODUCTION

There has been comparatively little attention paid in the past to the corynebacteria which are found in the bovine udder. With the exception of Corynebacterium pyogenes, these corynebacteria, the majority of which form a homogeneous group - Corynebacterium bovis -, were considered to be apathogenic and incapable of affecting udder or milk. Most of the bacteriological work on the prevention and cure of mastitis has been concerned with the eradication of streptococci and staphylococci, but there is an increasing number of cases of mastitis from which these organisms cannot be isolated. Mastitis is of continuing economic importance, in spite of the attempts made to eradicate streptococcal and staphylococcal infection. This disease may be responsible for an annual loss of £9-20 million due to the reduction of milk yield (Wilson, 1963, 1964 a and b) with an estimated depreciation in the market value of stock of £762,000 annually (West, 1966). As a result of surveys in England and Wales it has been found that in any one month 14% of dairy cows may suffer from clinical mastitis and at any one time 25% may suffer from subclinical mastitis (Wilson, 1964 b). A cow with clinical mastitis usually loses about 20% of the milk yield predicted for that lactation and O'Donovan, Dodd and Neave (1960) found that subclinical mastitis can depress the yields of milk, solids-not-fat and butter-fat by 10, 11 and 12% respectively.

The organisms commonly regarded as potential causes of mastitis (particularly clinical mastitis) are Staphylococcus aureus, Streptococcus agalactiae, Str. uberis, Str. dysgalactiae and (in

summer mastitis) Corynebacterium pyogenes. The predominating organism amongst these has changed over the years, Str. agalactiae being the organism which was most important before the introduction of antibiotics, but since the disease has been treated with antibiotics Staph. aureus has become the organism which, of the five mentioned, is most usually isolated from cases of mastitis. Thus, in 1942, a survey carried out from Weybridge found that Str. agalactiae was responsible for 44% of the clinical cases of mastitis, and Staph. aureus was responsible for 17% of the clinical cases, whereas in 1962 the figures were 4% and 34% respectively (Wilson, 1963).

The organisms which have been termed "low-grade pathogens" (Wilson 1963) have received little attention. Wall (1918) listed, in addition to streptococci, staphylococci and C.pyogenes, the following as causative organisms of mastitis: Escherichia coli, Mycobacterium tuberculosis, Actinomyces bovis and Sphaerophorus necrophorus. Hammer (1948) listed Aerobacter aerogenes, E.coli and Pseudomonas aeruginosa as being occasionally responsible for mastitis; Wilson and Miles (1955) briefly mentioned coliform bacteria, Sph. necrophorus and Ps.aeruginosa.

The difficulty of deciding which organisms are capable of causing mastitis results from the method of diagnosis of the disease. The onset of mastitis is characterized by the accumulation of leucocytes and the entry of blood constituents into the milk. The diagnosis of mastitis is based on the presence of clinical symptoms in the animal: changes in the composition of the milk toward that of blood (for example, rise in pH, increase in chloride content, the presence of catalase); the presence of "leucocytes" in the milk; and the detection of causal

organisms. Obviously mastitis is most readily diagnosed when clinical symptoms are present in the animal and when there are obvious physical changes in the milk (presence of clots, or the udder secretion being thin and watery, possibly yellow in colour, and possibly tinged with blood). When mastitis of this advanced type has been diagnosed the organisms isolated from the udder secretion are strongly suspected of causing the mastitic condition. Before the introduction of antibiotics streptococci were most frequently isolated from such cases, and staphylococci were the second most common organism. These organisms in particular therefore have gained a reputation as being the chief causative organisms of bovine mastitis. When subclinical and incipient mastitis was to be diagnosed before the development of clinical symptoms or gross physical changes in the milk, reliance was placed upon the presence of leucocytes in the milk, and the detection of organisms capable of causing mastitis (i.e. streptococci and staphylococci). In the event of causative organisms not being isolated, the condition has been termed "non-specific mastitis" (Malcolm and Campbell, 1946; Laing and Malcolm, 1956). The diagnosis of non-specific subclinical mastitis thus depended entirely on the detection of a high cell count in the milk. Total cell counts and differential cell counts distinguishing between leucocytes and epithelial cells or between different types of leucocytes (Blackburn and Macadam, 1954) have all been used for this purpose by different workers (Blackburn, Laing and Malcolm, 1955; Laing and Malcolm, 1956).

Thus there was made an implicit assumption that the streptococci

and staphylococci most frequently isolated from cases of clinical mastitis were the organisms also responsible for initiating the mastitic infection, since the absence of streptococci and staphylococci (and C.pyogenes) in milk showing a high cell count usually caused the sub-clinical mastitis to be called non-specific unless large numbers of coliforms, Ps.aeruginosa or certain other organisms recognized as low-grade pathogens were isolated. These last-named organisms were regarded as (low-grade) pathogens principally because they were not found in the healthy bovine udder. On the other hand, the organism very commonly found in the healthy (and mastitic) udder, and capable of being isolated from a very high proportion of samples of freshly drawn milk - namely C.bovis - was regarded as an apathogenic member of the normal flora of the teat-canal of no significance in the development of mastitis. Unfortunately the published surveys covering both the incidence of bovine mastitis and the organisms isolated from milk samples have usually suffered from the failure to report all the organisms isolated. Corynebacterium bovis is the organism most frequently omitted (e.g. Laing and Malcolm, 1956). Because of this selection of organisms to be reported there exists little evidence on the relative incidence, both qualitative and quantitative, of C.bovis in the udder secretions from normal udders compared with the secretions from cases of subclinical or clinical mastitis.

A survey conducted by Ochi and Katsube (1958) on 53 cows in 3 herds revealed that from the normal udder Staphylococcus was isolated from 83% and Corynebacterium was isolated from 51% of the samples

whereas in the case of the mastitic udder, Staphylococcus was isolated from 30% of the samples, and Corynebacterium was isolated from 20% of the samples (Streptococcus being isolated from mastitic udders in 75% of the cases). All the strains of Corynebacterium which they isolated from the normal udder were C.pseudodiphthericum (a species considered to include C.bovis within its wider definition) whereas of the corynebacteria isolated from cases of mastitis 70% were identified as C.pyogenes and 30% as C.pseudodiphthericum.

It is considered that the relationship of C.bovis to bovine mastitis deserves closer attention for two reasons: firstly the importance of C.bovis in the initial stages of mastitis has not been determined - the staphylococci and streptococci found so commonly in clinical mastitis could be secondary infections -, and secondly it is possible that certain of the changes in the methods of milk production have changed the susceptibility of the udder to the organisms which have previously been regarded as comparatively harmless - in particular the average milk yield of the dairy cow has increased by 70% since 1945, and machine milking has become the usual method of milking. The use of machine milking has resulted in an increase in the incidence of mastitis: a survey carried out in 1958-59 showed that 10.5% of cows milked by machine suffered from acute or mild mastitis compared with 6.0% of cows milked by hand (Report, 1964). Until very recently the possibility of C.bovis being of importance in mastitis has been dismissed by most of the workers in the field of bovine mastitis. There have however been one or two exceptions to this general lack of interest in C.bovis - for

example Little, Brown and Plastridge (1946) thought it questionable whether the harmlessness of the milk diphtheroids other than C.pyogenes could be taken for granted, and they recognized the need for more careful study of their effects in the udder. Very recently, since the inception of the present investigation, there has been an increasing interest in C.bovis and its role in bovine mastitis. The Central Veterinary Laboratory at Weybridge (Reports, 1965, 1966) is now recognizing C.bovis as a possible cause of clinical mastitis, and reports its recovery as the sole organism present in the udder secretion from a number of cows suffering from acute mastitis (Wilson, 1963, 1964a). Bruford and his colleagues (Bruford et al., 1965) reported that during the last few years C.bovis had been recovered from quite severe cases of mastitis as the sole isolate. Furthermore in certain herds it had been shown to produce a marked increase in the cell count of the milk, and sometimes more than 60% of the cows in a herd were involved. In an admittedly small-scale inoculation experiment Cobb and Walley (1962) obtained symptoms of mastitis by the intramammary inoculation of live and dead cultures of C.bovis and of its culture filtrates.

There have been comperatively few detailed studies of C.bovis since Evans (1916, 1917) described the characteristics of Bacillus abortus var. lipolyticus which she later considered (Breed, Murray and Smith, 1957) to be identical with C.bovis. The characteristics which she described were confirmed by Bendixen (1933) whose "Type A udder corynebacteria" corresponded with C.bovis. The organism seemed to be biochemically inactive apart from a slight tendency to the production of

acid in the cream layer of whole milk. Because of the apathogenicity and biochemical inactivity of C.bovis it has commanded little attention subsequently. The few investigations which have included a study of C.bovis (for example those of Brooks and Hucker, 1944; Breuillaud and Michel, 1962; and Ochi and Zaizen, 1940, whose C.pseudodiphthericum Type II was considered by Ochi (Katsube, 1964, personal communication) to include C.bovis) have tended to confirm this biochemical inactivity, with the weak lipolytic ability of the organism being the only positive characteristic. Even this characteristic has been rather inadequately demonstrated (Black, 1941). Abd-el-Malek and Gibson (1952), referring to Bacterium lipolyticum Evans (sic) stated that ". . . since this organism is biochemically inactive in milk, and since the colonies which it might occasionally produce on milk-count agars are so small that they would easily escape detection, its occurrence in milk appears to be of little practical interest." Consequently these two workers did not study the heat-sensitive diphtheroids in detail.

It seemed desirable to examine C.bovis in greater detail than had been attempted hitherto. Firstly such work may help to throw some light on the reasons for the restricted habitat of C.bovis. In addition the biochemical reactions of C.bovis required clarification, since in previous investigations either the recipes of the basal media used for biochemical tests were not given or the basal media used (e.g. nutrient agar, peptone water etc.) were unsuitable for its growth although the enhancement of growth of C.bovis by serum was described by Evans (1916).

Isolates of coryneform bacteria were obtained from samples of

freshly-drawn bovine milk submitted to the Bacteriology Department of the West of Scotland Agricultural College for routine examination for mastitis. The samples were examined by culture of the organisms present in the milk on blood agar and on serum agar, and the results of bacterial culture were compared with the presence of clinical mastitis, the physical appearance of the milk and the total bovine cell count. Information on these three aspects was obtained from the specialist Advisory Officer concerned with the routine investigation of the milk samples.

The cultural characteristics of the isolates and of a range of type cultures of Corynebacterium were examined, primarily to elucidate the nature of the cultural requirement of C.bovis for serum. The biochemical reactions of the isolates and type cultures were subsequently examined using basal media capable of supporting the growth of the nutritionally exacting corynebacteria C.bovis, C.pyogenes and C.diphtheriae.

In order to further elucidate the nature of the requirement of C.bovis for serum, and perhaps to help in the elucidation of the reasons for the predilection of C.bovis for the bovine udder, the growth of C.bovis was studied in a range of chemically defined media.

As a result of the difficulty in the interpretation of the results obtained with C.bovis on lipolysis test media, an attempt was made to isolate the lipolytic and Tween-hydrolysing enzymes of C.bovis and to study their substrate preferences by the use of zone electrophoresis.

SOURCE OF CULTURES STUDIED

Experimental methods

Milk samples from 14 different farms were examined by direct plating of samples obtained from individual quarters of bovine udders on (a) blood agar (pH 7.2) containing (% w/v): peptone (Oxoid), 1.0; Lab-Lemco beef extract (Oxoid), 1.0; sodium chloride, 0.5; agar, 1.2; to which was added after sterilization 5% (v/v) of oxalated horse blood (Burroughs Wellcome and Co., London), and (b) a tryptose serum agar (pH 7.2) containing (% w/v): tryptose (Oxoid), 1.0; Lab-Lemco beef extract (Oxoid), 0.3; sodium chloride, 0.5; agar, 1.2; to which was added after sterilization 5% (v/v) of sterile bovine serum (Oxoid). The blood or serum was added aseptically to the liquefied medium at ca. 50°C just before pouring the plates. The examination was made roughly quantitative by the use of a graduated closed wire loop (4 mm internal diameter) which delivered approximately 0.01 ml of milk. The milk samples were streaked over the surface of the plates which had been previously poured and dried. The four quarter samples from one animal were streaked in separate sections of one plate. The plates were incubated at 37°C and examined after 24 and 48 hours; they were also examined after 3 days at 37°C followed by 3 days at 25°C. The colonial characteristics of the isolates were observed and microscopic examinations were carried out on Gram-stained heat-fixed smears.

The milk samples were also examined for total bovine cellular content using the Breed's smear technique and Newman's stain*

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*Newman's stain

Methylene blue	1.0 g
Ethanol, 95%	54 ml
Tetrachlorethane	40 ml
Glacial acetic acid	6 ml

The ethanol was added to the tetrachlorethane and heated in a water bath at 70°C. The methylene blue was then added and shaken until dissolved. The acetic acid was added to the cool solution and the mixture filtered.

(American Public Health Association 1960). The milk sample (0.01 ml) was spread over an area of 1 cm^2 on a clean glass slide, and dried by placing the slide on a hot plate at 55°C. The slide was placed in a coplin jar containing Newman's stain for 2 minutes. The slide was then removed from the stain and allowed to dry in air. When dry the smear was washed by placing the slide in a coplin jar containing water, the jar being gently agitated during the washing procedure. After washing the smear was once again allowed to dry in air.

After determining the diameter of the microscope field using the oil immersion objective and a slide micrometer, the number of bovine cells in up to 30 randomly selected fields was determined. In the case of smears in which the cells were uniformly distributed, the number of fields counted depended upon the average number of cells present per field, in a manner similar to that suggested by Wilson (1935).

Average number of cells per field	Number of fields counted
0 - 3	30
4 - 6	15
7- 10	10
11 - 17	6
18 - 30	3
More than 30	1

If the cells were not distributed uniformly throughout the smear, 30 fields were counted.

Results and isolates obtained from bovine milk samples

One thousand three hundred and thirty four (1,334) quarter samples were examined from 335 cows on 14 farms over a period of two years (Table 1).

On the basis of the bovine cell count the 1,334 quarter samples

TABLE 1

FARMS FROM WHICH SAMPLES WERE OBTAINED

Location of Farm	Date of examination	Number of quarter-samples	Number of cattle
High Blantyre, Lanarkshire.	1/ 5/62	72	18
	15/ 5/62	76	19
	3/ 7/62	76	19
Eaglesham, Renfrewshire.	8/ 5/62	181	46
	15/ 5/62	24	6
Auchterarder, Perthshire.	5/ 7/62	91	23
Coatbridge, Lanarkshire	9/ 7/62	8	2
Castle Douglas, Kirkcudbright.	25/ 7/62	112	28
Kilkerran, by Maybole, Ayrshire.	31/ 8/62	84	21
	26/10/62	36	9
Denny, Stirlingshire.	7/ 9/62	16	4
Baillieston, Lanarkshire.	15/10/62	8	2
Campbelltown, Argyllshire.	17/10/62	44	11
Crocketford, Kirkcudbrightshire.	25/10/62	84	21
Bute	10/12/63	72	18
Strathaven, Lanarkshire.	27/ 2/64	135	34
Lenzie, Lanarkshire	30/ 4/64	40	10
Dunoon, Cowal, Argyllshire.	30/ 4/64	175	44
	TOTAL	1,334	335

TABLE 2: CELL COUNTS OF, AND ISOLATES FROM BOVINE MILK SAMPLES

TABLE 2: CELL COUNTS OF, AND ISOLATES FROM, BOVINE MILK SAMPLES

State of quarter	Number of quarter samples tested	Number from which corynebacteria were isolated	Number from which only corynebacteria were isolated	Number from which *C.bovis* were isolated	Number from which only *C.bovis* were isolated	Number from which staphylococci were isolated	Number from which streptococci were isolated	Number in which staphylococci and/or streptococci were present but corynebacteria were absent	Number in which organisms other than corynebacteria, staphylococci or streptococci were found		Number from which no organisms were isolated
									Alone	With staphylococci streptococci or corynebacteria	
Normal (< 250,000 bovine cells per ml)	710	451	322	441	313	199	12	106	0	36	153
Suspect (250,000 to 500,000 bovine cells per ml)	147	107	48	107	48	70	8	24	1 unknown, 1 *Sarcina*	8	14
Positive, subclinical mastitis (> 500,000 cells per ml)	389	245	91	243	89	255	61	107	4 coliforms, 1 mould.	27	32
Clinical (with positive cell count)	49	35	11	35	11	28	8	8	0	3	6
Clinical mastitis (with negative cell count)	3	3	2	3	2	1	0	0	0	0	0
Not known	36	18	16	18	16	6	0	14	0	0	4
Total numbers:	1,334	859	490	847	479	559	89	259	7	74	209

were grouped as suggested by Laing and Malcolm (1956): with a cell count of less than 250,000 per ml, the milk was regarded as being derived from a normal, healthy quarter; a cell count of 250,000 - 500,000 per ml was considered to be possibly derived from a quarter in which there was mastitis; a cell count of more than 500,000 per ml was considered indicative of mastitis (a subclinical mastitis in the absence of any manifest abnormality of milk or udder). In addition the cases of clinical mastitis were also noted. The isolates on the blood agar and serum agar plates were broadly identified on the basis of colonial appearance and microscopic examination of Gram-stained smears. The results are set out in Table 2.

Frequently, slow-growing colonies of corynebacteria were detected after 3 days at 37°C followed by 3 days at 25°C, where they had not been evident after 3 days at 37°C.

From these samples 142 strains of corynebacteria were obtained in pure culture and their cultural and biochemical characteristics examined. The isolates were taken both from normal udders and quarters showing evidence of clinical or subclinical mastitis as shown in Table 3. In [illegible] cases in which only

TABLE 3

THE ORIGIN OF THE CORYNEBACTERIUM STRAINS EXAMINED

TABLE 2

THE ORIGIN OF THE CORYNEBACTERIUM STRAINS EXAMINED

Cell count/ml of milk sample	State of udder quarter	No. of isolates
< 250,000	Normal	66
250,000-500,000	Suspect	18
> 500,000	Positive (subclinical mastitis)	33
—	Clinical mastitis	8
Not known	Not known	16

corynebacteria were detected or in which the corynebacteria greatly outnumbered any other micro-organisms present.

The examination of milk samples from animals other then cows

In addition to the examination of bovine milk samples detailed in Table 2, 32 "half-samples" of caprine milk (from 13 different goats), one equine milk sample, and 9 human milk samples (from 9 different women) were similarly examined by plating 0.01 ml amounts on blood agar and serum agar. No corynebacteria were detected in the samples of caprine milk. Corynebacteria were present in the equine milk sample and three colonies were selected (strains 170, 171, and 172) for further study and for comparison with the corynebacteria isolated from the bovine milk. In only one human milk sample were corynebacteria detected and isolated (strain 173).

Type cultures used for comparative purposes

The cultural, morphological and biochemical characteristics of the isolates were compared with those of the following type cultures:

Corynebacterium bovis NCTC (National Collection of Type Cultures) 3224,

C.hofmannii NCTC 231 C.flavidum NCTC 764 C.ovis NCTC 3450

C.diphtheriae NCTC 3985 C.pyogenes NCTC 6448 C.xerosis NCTC 7243 and NCTC 9755.

C.renale NCTC 7448 C.ulcerans NCTC 7910

Microbacterium lacticum NCIB (National Collection of Industrial Bacteria) 8540 and 8541.

Maintenance of stock cultures

Stock cultures were kept on Loeffler's serum slopes (Oxoid) in screw-capped ½ oz (bijou) bottles, incubated for 2 days at 37°C after inoculation, and subsequently kept refrigerated. The stock cultures

were subcultured once every 20 weeks on to tryptose serum agar plates and each fresh stock culture prepared by inoculating Loeffler's serum slopes with a wire loop which had been charged with bacteria from a large number of colonies in order to avoid the inadvertent selection of mutants.

Discussion of the results of the examination of the bovine milk samples

In 91 of the 389 samples diagnosed (on the basis of cell count) as originating from quarters with a subclinical mastitis C.bovis was the only organism isolated. In only 8 of the 52 cases of clinical mastitis found in the 1,334 samples was Streptococcus isolated. Staphylococci were isolated from 29 of the 52 samples, but this included both Staphylococcus aureus and Staph.epidermidis which were not differentiated. Corynebacteria, however, were isolated from 38 of the 52 samples and were the sole organisms isolated from 13 of the quarters with clinical mastitis. In some of these samples the corynebacteria were detected in numbers exceeding 50,000 per ml (the maximum count it was possible to distinguish by a surface counting method in which 0.01 ml was spread over one quarter of an agar plate). These results would suggest that C.bovis is capable of causing clinical mastitis, as well as causing subclinical mastitis. For many years the majority opinion was that C.bovis was a harmless parasite in the normal udder but the apparently increasing frequency with which it is being isolated as the sole organism from udders in which clinical mastitis is manifest has occasioned its inclusion amongst the mastitis-causing organisms (Cobb and Walley, 1962; Wilson, 1964a; Report, 1965). The results obtained in the present work suggest that in the absence of a more profound knowledge of the causality in

which mastitis and the proliferation of the udder flora are inter-related factors C.bovis should be regarded as a potential mastitis organism, in addition to the possibility of its being the primary infective organism in incipient mastitis. In this case its occurrence in 847 of the 1,334 quarter-samples, together with the presence of staphylococci and/or streptococci in 259 quarters in which corynebacteria were not detected means that at least 1,106 of the 1,334 samples (82.9%) contained potentially pathogenic organisms.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS

Introduction

The microscopical appearance of Corynebacterium bovis is typical of the genus Corynebacterium described by Breed, Murray and Smith (1957) as consisting of straight to slightly curved non-sporing rods, with irregularly stained segments, and sometimes containing granules. Club-shaped swollen ends occur frequently, and snapping division produces angular and palisade arrangements of the cells. The organisms are Gram-positive but may occasionally stain Gram-negative with Gram-positive granules. The size is generally 0.5 to 0.7 μ by 2.5 to 3.0 μ.

The cultural characteristics of C.bovis, the best of the early descriptions of which were by Evans (1916) and Bendixen (1933), include an enhancement of growth by the addition of serum to the medium. The colonies are non-pigmented and greyish on serum agar but both Evans and Bendixen noted that in old cultures that had been incubated for a week or so there was a dark brown discoloration of the serum agar. If growth occurred in nutrient agar, nutrient broth or nutrient gelatin to which serum had not been added, it was extremely sparse and that only with massive inocula. Both workers also noted the very characteristic growth obtained in agar shake cultures in which growth occurred in a zone about 1 mm thick between 3 and 6 mm below the surface.

At an early stage in the present work the cultural characteristics of the isolates were studied in detail since it was felt that the

reported biochemical inactivity could be due to the basal media used by previous workers offering less than optimum conditions for growth of the organisms.

Materials and methods

The growth of the stock cultures was studied on the media detailed below, by inoculating from 48-hour tryptose serum agar cultures. Solid media were inoculated by streaking on to poured dried plates except where otherwise stated.

Nutrient broth

Peptone 10.0 g	Lab-Lemco beef extract (Oxoid) 10.0 g
Sodium chloride 10.0 g	Distilled water 1 litre

The reaction of the medium was adjusted to pH 7.2. The medium was then distributed in 2.5 ml amounts in 4 x $\frac{1}{2}$ inch test tubes and sterilized by autoclaving at 121°C for 15 minutes.

Peptone water

In 1 litre of distilled water were dissolved tryptone (Oxoid), 10.0 g; sodium chloride, 5.0 g; the pH was adjusted to 7.2. The medium was dispensed in 2.5 ml amounts in 4 x $\frac{1}{2}$ inch test tubes and sterilized by autoclaving at 121°C for 15 minutes.

Panmede broth

This consisted of Panmede liver digest (Paines & Byrne Ltd., Greenford, Middlesex), 0.8% (w/v) in distilled water, adjusted to pH 7.5. Sterilization was by filtration, and the sterile broth dispensed aseptically in 2.5 ml amounts in screw-capped $\frac{1}{4}$ oz (bijou) bottles. The caps were screwed on tightly and the bottles immersed in a water bath at 63°C for 30 minutes.

Panmede albumin broth

This was prepared by the aseptic addition of 0.28 ml of a

filter-sterilized 3% (w/v) albumin (bovine serum albumin fraction V, Armour Pharmaceutical Co. Ltd., Eastbourne) solution to each 2.5 ml amount of Panmede broth, to give a final concentration of albumin of 0.3% (w/v).

Panmede glucose broth

To each 2.5 ml amount of Panmede broth was added 0.28 ml of a sterile 10% (w/v) glucose solution, to give a final concentration of glucose of 1% (w/v).

Panmede yeast extract broth

This was prepared by the aseptic addition of 0.28 ml of a filter-sterilized 5% (w/v) yeast extract (Oxoid) solution to each 2.5 ml amount of Panmede broth, to give a final concentration of yeast extract of 0.5% (w/v).

Tryptose serum broth

Tryptose (Oxoid) 10.0 g Lab-Lemco beef extract (Oxoid) 3.0 g

Sodium chloride 5.0 g Distilled water 1 litre

The pH was adjusted to 7.2. The medium was dispensed in 2 ml amounts in 4 x $\frac{1}{2}$ inch test tubes, and sterilized at 121°C for 15 minutes. After sterilization and when cool, sterile bovine serum (Oxoid) was added aseptically to a final concentration of 5% (v/v).

Nutrient agar

Nutrient agar consisted of nutrient broth to which was added 15.0 g of agar per litre of broth.

Tryptose lemco agar

The medium used was Tryptose Blood Agar Base (Oxoid), sterilized

by autoclaving at 121°C for 15 minutes.

Blood agar

Oxalated horse blood (Burroughs Wellcome and Co., London) was added aseptically to Tryptose Blood Agar Base (Oxoid), previously melted and cooled to 45°C, to a final concentration of 5% (v/v).

Blood agar + sodium polyanethol sulphonate

von Haebler and Miles (1938) suggested the addition of 0.05% of sodium polyanethol sulphonate ("liquoid") to blood-containing media used for the growth of fastidious pathogens to counteract the bactericidal activity of the blood. A stock 2% solution of sodium polyanethol sulphonate (Koch-Light Laboratories, Colnbrook, Bucks.) was prepared in distilled water and sterilized by filtration. To 100 ml of molten Tryptose Blood Agar Base (Oxoid) cooled to 45°C, 5 ml of sterile oxalated equine blood (Burroughs Wellcome and Co.) and 2 ml of the sterile sodium polyanethol solution were added aseptically, thoroughly mixed and plates poured.

Bovine serum agar, calf serum agar and horse serum agar

These were prepared in a manner similar to blood agar, by substituting sterile bovine serum (Oxoid), sterile calf serum (Oxoid), or sterile horse serum (Oxoid) for the oxalated horse blood.

Skim milk agar

The skim milk was prepared by reconstituting skim-milk powder (Oxoid) which was sterilized by steaming for 30 minutes on 3 successive days. This was then added to Tryptose Blood Agar Base (Oxoid) which had been melted and cooled to 50°C, to give a final concentration of

reconstituted skim milk of 5% (v/v).

Glucose agar and glucose serum agar

Glucose agar was prepared by dissolving 1.0 g of glucose in 100 ml of molten Tryptose Blood Agar Base (Oxoid), and sterilizing the medium by autoclaving at 115°C for 15 minutes. Glucose serum agar was prepared by adding aseptically sterile bovine serum (Oxoid) to molten glucose agar at 45°C, to a final concentration of 5% (v/v). In addition to poured plates, the glucose serum agar was also used to prepare shake cultures, the medium being dispensed in 10 ml amounts in 6 x $\frac{5}{8}$ inch test tubes. The inoculum was added to a tube of molten sterile medium at 45°C, mixed thoroughly (avoiding the inclusion of air bubbles) and the medium allowed to solidify with the tube in the upright position.

Charcoal agar

To 100 ml of Tryptose Blood Agar Base (Oxoid), 0.4 g of purified activated charcoal (Oxoid) was added with thorough mixing. The medium was sterilized by autoclaving at 121°C for 15 minutes.

Butter-fat agar

The butter-fat was prepared from unsalted butter by melting the butter at 50°C, the butter-fat then being removed from the curds and distributed into screw-capped 1 oz bottles in 10 ml amounts. Sterilization was effected by autoclaving at 121°C for 15 minutes. The medium was prepared by adding aseptically 5 ml of molten sterile butter-fat to 100 ml of molten Tryptose Blood Agar Base (Oxoid), and emulsifying by vigorous hand-shaking immediately before pouring the plates.

Egg-yolk agar

This was prepared by adding 1 g of sodium chloride per 100 ml of Tryptose Blood Agar Base (Oxoid) before sterilization by autoclaving at 121°C for 15 minutes. After sterilization the medium was cooled to 45-50°C and 10 ml of Concentrated Egg-yolk Emulsion (Oxoid) were added aseptically to, and mixed with, each 100 ml of medium immediately before pouring the plates. The medium was also prepared without sodium chloride.

Loeffler's serum slopes

Ready-prepared Loeffler's serum slopes (Oxoid) in screw-capped ½ oz (bijou) bottles were used.

Incubation of cultures in a carbon dioxide-enriched atmosphere

In addition to the above cultures which were incubated aerobically, cultures inoculated on tryptose lemco agar, bovine serum agar, glucose agar, charcoal agar, egg-yolk agar, and butter-fat agar were also incubated in air with an increased carbon dioxide content. The inoculated plates were placed in "half-size" biscuit tins (dimensions approximately 9 x 8½ x 5 inches) which had been made airtight by sealing the seams with a sealing compound. The carbon dioxide was generated by adding 1.2 g of marble chips to 15 ml of 2N hydrochloric acid contained in a glass bottle, to give a carbon dioxide concentration of between 4.6 and 5.9%. The lid of the tin was then immediately replaced and sealed on.

Survival at 72°C

Heat resistance tests of a qualitative nature were carried out by

the method of Abd-el-Malek and Gibson (1948). A heavy inoculum from a 3 day serum agar culture was placed in a 6 x $\frac{5}{8}$ inch test tube containing 5 ml of sterile reconstituted skim milk (Oxoid) that had previously been sterilized by autoclaving at 121°C for 5 minutes followed by steaming for 30 minutes on each of two consecutive days. Care was taken in the inoculation of the skim milk to avoid contaminating the side of the tube with the culture or wetting it with the milk. The inoculum and the skim milk were well mixed and a loopful removed and streaked on the surface of a plate of egg-yolk agar.

The inoculated tubes and a control tube of skim milk into which a thermometer had been inserted through the cottonwool plug were placed in a thermostatically controlled water bath fitted with a mechanical shaker (manufactured by A. Gallenkamp & Co. Ltd., London E.C.2).

In order that the sampling could be carried out at regular 5 minute intervals, the immersion of the tubes in the water bath was staggered with 30 seconds being allowed between insertion of consecutive tubes, the tube containing the thermometer being the first tube to be placed in the bath. The tubes were shaken at the slowest speed (ca. 2 oscillations per second).

Thirty seconds from the moment when the skim milk in the control tube attained 72°C, a loopful of the first inoculated skim milk to be placed in the water bath was removed from the tube and streaked across egg-yolk agar. The other tubes were similarly sampled at 30-second intervals. These samples represented a zero time heat resistance test

at 72°C. This procedure was then repeated, samples being withdrawn from each tube every five minutes up to a maximum of 35 minutes.

Egg-yolk agar was chosen as the recovery medium since it is known that rich media give the highest recovery rates in heat resistance tests (Nelson, 1943), and egg-yolk agar allowed at least as good growth of the organisms as was obtained on serum agar without the batch variability that sometimes occurred with the latter medium.

The streaked plates of egg-yolk agar were incubated for 5 days at 37°C, with the exception of the plates carrying Microbacterium lacticum NCIB 8540 and 8541 which were incubated at 30°C. After incubation the amount of growth was assessed visually.

Results

One hundred and twenty-seven of the 142 isolates corresponded morphologically and culturally to C.bovis both by comparison with the description in Bergey's Manual (Breed et al., 1957) and with the type culture C.bovis NCTC 3224. This identification was confirmed by the biochemical characteristics of the isolates when these were compared with those of C.bovis NCTC 3224 (See Section D). Although Jayne-Williams and Skerman (1966) over a 2 year period isolated from bovine udder quarters a number of organisms corresponding to C.ulcerans none of the corynebacteria isolated in the present work appeared to be identical with C.ulcerans either culturally or biochemically. Jayne-Williams and Skerman suggested that C.ulcerans could be mistakenly identified as a coccus on primary isolation plates which usually required 3 day incubation at 37°C for colonies of corynebacteria to become visible. This seems to be an unlikely explanation for the failure in the present work to isolate strains of C.ulcerans since firstly many colonies on the primary isolation plates consisting of organisms showing staphylococcal or streptococcal morphology were subcultured, and none subsequently exhibited a corynebacterial morphology, and secondly microscopic examination of cultures of C.ulcerans NCTC 7910 that had been incubated for 4 days at 37°C revealed coccobacilli and short rods that were considered to be typical of Corynebacterium and not capable of being mistaken for cocci.

Of the remaining 15 isolates, eight (strains 7, 17, 39, 46, 47, 48, 49 and 50) were tentatively identified as Nocardia on the basis of

morphological and cultural characteristics, although no further identification was attempted. Seven of these 8 nocardiae had been isolated from milk samples obtained from the same farm, and these proved to be identical in all their cultural characteristics and biochemical reactions. Five strains (12, 13, 14, 15 and 33) of the 15 isolates resembled Microbacterium, and these 5 strains also originated from milk samples obtained from a single farm. The remaining 2 strains (103 and 154) of these 15 isolates were possibly Corynebacterium but they did not correspond with any of the type cultures examined.

The 127 strains of C.bovis proved to be incapable of growth on nutrient broth or peptone water. They failed to grow in Panmede broth either without supplementation or with the addition of albumin, yeast extract or glucose. Similarly no growth was obtained on nutrient agar, tryptose lemco agar, glucose agar, charcoal agar or skim-milk agar. Only moderate growth was obtained on blood agar or blood agar containing sodium polyanethol sulphonate but moderate to profuse growth occurred on serum agar.

The variation in the amount of growth produced on media made from different batches of serum of the same type was considerable, and as great as the variation in growth observed on media containing horse, calf or bovine serum. Extremely good growth was obtained on egg-yolk agar and butter-fat agar without the variability noticed in the case of serum agar. Incubation in a carbon dioxide-enriched atmosphere had no apparent effect. The growth obtained in glucose serum agar shake cultures was exactly as described by Evans (1916) and Bendixen (1933),

with the growth occurring in the top 5 mm of the medium, particularly in a zone 1-2 mm thick which occurred just beneath the surface of the medium. The isolates of "nocardiae" and Microbacterium were also obligately aerobic with the growth restricted to the top 5 mm of the medium, but subsurface zone formation did not occur. These isolates also differed from the C.bovis isolates in their growth on the other media. The nocardial isolates grew very well in nutrient broth, peptone water, the Panmede broths (with best growth in Panmede glucose broth) and tryptose serum broth with the formation of a pellicle and granular sediment. They gave good growth on nutrient agar, tryptose lemco agar, charcoal agar, glucose agar, and egg-yolk agar, but they grew only moderately well on butter-fat agar; incubation in a carbon dioxide-enriched atmosphere had no apparent effect. In contrast the isolates of Microbacterium grew in nutrient broth, peptone water, the Panmede broths and tryptose serum broth with the development of a non granular sediment and with no pellicle formation. They gave moderate growth on nutrient agar and tryptose lemco agar, the addition of glucose increasing the amount of growth. Good growth was obtained on serum agar, butter-fat agar and egg-yolk agar, and there was little growth on charcoal agar. Incubation in a carbon dioxide-enriched atmosphere gave somewhat better growth than that obtained in air.

All isolates gave good growth on Loeffler's serum slopes. The optimum temperature of all isolates appeared to be 37°C.

The colonial appearances of all the isolates of C.bovis were very similar. On serum agar the colonies were greyish-white, matt, and of a crumbly consistency. The dark brown discoloration of serum agar

observed by Evans (1916) and Bendixen (1933) to occur in old cultures was occasionally noticed but proved to be an inconstant characteristic. The growth on egg-yolk agar and Loeffler's serum slopes was more cream-coloured. A more positive pigmentation occurred on butter-fat agar, the growth being white, cream or yellow depending on the strain. This pigmentation was found during the investigation of the biochemical characteristics of the organisms to be correlated with the ability of the organisms to hydrolyse the butter-fat.

Growth of the three isolates from equine milk and the single isolate from human milk was very sparse on all the media described irrespective of enrichment by carbon dioxide or of the incubation temperature. The use of stab cultures and shake cultures revealed no microaerophilic tendency. Subculture of two of the equine diphtheroids and the human diphtheroid very soon failed.

The optimum temperature for growth of the type cultures of Corynebacterium was between 30 and 37°C inclusive. The type cultures of Microbacterium lacticum grew poorly at 37°C, although this was dependent upon the medium used, and the optimum temperature for growth was ca. 30°C.

The type cultures of C.flavidum, C.ovis, C.diphtheriae, C.xerosis, C.renale, C.ulcerans and Microbacterium lacticum grew well on a nutrient agar (Oxoid Tryptose Blood Agar Base) without the addition of blood, serum, egg-yolk or Tween. The type cultures of C.hofmannii, C.bovis and C.pyogenes grew poorly or not at all on nutrient media such as Tryptose Blood Agar Base, and the addition of serum was necessary for good growth to be obtained. Corynebacterium hofmannii and C.bovis

were also able to grow on various Tween agars and egg-yolk agar (see Appendix to Section D), but C.pyogenes grew only sparsely on the Tween agars and egg-yolk agar. The growth characteristics of the type cultures were similar when the plates were incubated in an atmosphere containing about 5% of carbon dioxide, with the exception of Microbacterium lacticum NCIB 8540 and 8541, which were unable to grow on Tryptose Blood Agar Base under these conditions except when serum or egg-yolk was added. Both strains of M.lacticum also tended to be inhibited on charcoal agar, whereas the other type cultures were unaffected by the addition of charcoal to the basal medium.

Microbacterium lacticum NCIB 8540 was able to survive for 35 minutes or more at 72°C as assessed by the qualitative method of Abd-el-Malek and Gibson (1948). The other type culture strain of M.lacticum, NCIB 8541, survived for 10 minutes at 72°C with very few colonies growing from the samples taken after 15, 20, 25 and 30 minutes, no viable organisms being detected after 35 minutes exposure. The microbacterial isolates (strains 12, 13, 14, 15 and 33) survived 15 minutes at 72°C, but no viable organisms were detected after exposure for periods longer than 15 minutes. No other isolates or type cultures survived exposure at a temperature of 72°C, with the exception of a few strains of the C.bovis isolates (for example, strain 159) which produced one or two colonies only after 5 minutes at 72°C.

Discussion and conclusions

In the primary isolation of the 127 strains of C.bovis, milk samples were plated both on blood agar and on serum agar. Numbers and types of isolates obtained on these two media were very similar, and the colony size of the corynebacteria on primary isolation was not significantly different on the two media. Nevertheless when pure cultures of the isolates were streaked on to blood agar and serum agar, the growth on serum agar was substantially better than that obtained on blood agar. The most likely explanation for these results is that blood agar is somewhat inhibitory toward C.bovis but the organism is protected during the primary isolation by being present on the surface of the medium in a streak of milk, the protection perhaps being afforded by milk-fat globules. The bactericidal action of blood has been long recognized, von Haebler and Miles (1938) finding that the addition of the anticoagulant sodium polyanethol sulphonate to blood-containing media enhanced the growth of Diplococcus pneumoniae and Brucella melitensis. The addition of the sodium polyanethol sulphonate to blood agar did not enhance the growth of any of the corynebacteria examined, in the present work.

The growth of the C.bovis isolates on serum agar, together with the complete absence of growth on nutrient agar or tryptose lemco agar except when very heavy inocula were used, suggested that C.bovis has a nutritional requirement that is satisfied by the addition of serum to the medium. Whatever this nutritional requirement might be it would also appear to be satisfied by the addition of egg-yolk emulsion or

butter-fat to the medium. There is, however, an alternative explanation: that the serum, egg-yolk or butter-fat is rendering inactive some bactericidal or bacteriostatic compound already present in the basal medium. Several instances of this type of action have been reported for a variety of micro-organisms, including Corynebacterium. For example, Kodicek (1949) has described the bacteriostatic effect of unsaturated fatty acids on Gram-positive bacteria, noting that this bacteriostatic action can be prevented by the addition of a surface active agent such as lecithin. Hutner (1942) described the inhibition of Erysipelothrix and Listeria by oleic acid which nevertheless became non-inhibitory in the presence of 0.3%saponin. In this case Hutner found that the serum requirement of Erysipelothrix rhusiopathiae was satisfied by addition of oleic acid to the medium provided that the oleic acid was rendered non-inhibitory by saponin. Other compounds that have been found effective in detoxifying fatty acids include albumin (Davis and Dubos, 1947; Johnson and Gary, 1963), Tween 40 (Williams, Broquist and Snell, 1947) and activated charcoal (Pollock, Wainwright and Manson, 1949). It has been shown that ordinary nutrient media may contain sufficient free fatty acid to exert an inhibitory effect upon certain very sensitive organisms, the fatty acids being derived either from the meat extract or the peptone (Pollock et al, 1949), from agar (Pollock, 1949) or from heat sterilization of the media in containers plugged with cotton wool (Pollock, 1949; Hart, Lovelock and Nash, 1962). The inability of the C.bovis isolates to grow in Panmede albumin broth or on a tryptose lemco agar containing 0.4% activated

charcoal suggeststhat in this case the serum, egg-yolk or butter-fat is actually satisfying a nutritional requirement. This of course does not necessarily rule out the possibility that the nutritional requirement is for a fatty acid, if the fatty acid is required in concentrations greater than the amounts found in nutrient agar, tryptose lemco agar and similar media. For example, Silliker, Deibel and Fagan (1963) have reported isolating corynebacteria that require unsaturated fatty acid (as oleate) in concentrations possibly as high as 0.5%.

The use of empirical media in nutritional studies makes it extremely difficult to determine the true requirements of an organism, and in order to study the possible reasons for the requirement of C.bovis for serum, egg-yolk or butter-fat, an investigation was undertaken into the growth of two typical isolates in chemically defined media (see Section E).

The absence of any effect on the growth of C.bovis by a carbon dioxide-enriched atmosphere is interesting since freshly drawn bovine milk contains approximately 6.6 ml of dissolved carbon dioxide per 100 ml of milk, measured at $0^{o}C$ and 760 mm Hg pressure (Ling, Kon and Porter, 1961). Nevertheless these results do not exclude the possibility that the high carbon dioxide content of milk is important in establishing C.bovis in the udder, since some bacteria, although not possessing a continuing requirement for carbon dioxide, require its presence in order to grow from small inocula (Woods and Foster, 1964).

In view of the heat resistance of the five strains 12, 13, 14, 15 and 33, together with their close resemblance both morphologically and

culturally to the type cultures of Microbacterium examined, these organisms are tentatively identified as Microbacterium, although they differed in having an optimum growth temperature around 37°C, whereas the cultures of M.lacticum NCIB 8540 and 8541 had optimum temperatures for growth of 30°C and usually grew poorly, if at all, at 37°C. Thomas and Thomas (1955) observed that very few strains of Microbacterium were able to form readily visible colonies within 48 hours at 37°C.

BIOCHEMICAL REACTIONS

Introduction

Since Evans (1916) reported that Bacillus abortus var. lipolyticus had no effect on gelatin, nitrate, urea or asparagine, and did not attack carbohydrates and related compounds, numerous workers have similarly reported Corynebacterium bovis to be relatively biochemically inactive (see for example, Bendixen, 1933; Ochi and Zaizen, 1940; Black 1941; Breuillaud and Michel, 1962). The only positive biochemical reactions noted by these workers concerned the action of C.bovis on milk: it appeared to produce an alkaline reaction in litmus skim milk (probably due to the production of ammonia from proteins or amino acids), but produced acid from milk fat and caused rancidity. The reaction in litmus milk thus depended upon whether the medium used was litmus whole milk, when acid production and rancidity in the cream layer was reported (Evans, 1916; Bendixen, 1933) or litmus skim milk, when an alkaline reaction was noted (Breed et al, 1957). Cobb (1963) however, has reported an alkaline reaction in litmus whole milk. Although it had been reported that C.bovis could cause rancidity in cream (Evans, 1916; Bendixen, 1933; Black 1941) the only evidence for lipolytic activity obtained using biochemical test media was the observation by Black (1941) that C.bovis was weakly lipolytic when tested on tributyrin agar.

In view of the cultural requirements of C.bovis, it seemed possible that the biochemical inactivity resulted from the inability of the organism to grow in the basal media used, and therefore in the present work the biochemical reactions of the isolates were examined using

serum-containing media whenever possible.

Materials and Methods

Before inoculation, the biochemical test media were incubated for 48 hours at 37°C followed by 48 hours at 25°C to detect and eliminate any tubes or plates that were contaminated.

Cultures were prepared for biochemical tests by streaking on plates of bovine serum agar and incubating for 2 days at 37°C (with the exception of the type cultures of Microbacterium lacticum which were incubated at 30°C). Inocula were obtained by taking a surface smear of a large number of colonies, to ensure that isolated mutants were not obtained in pure culture. Inoculated test media were usually incubated at 37°C except in the case of type cultures of M.lacticum which were incubated at 30°C, or 30°and 37°C.

It was not possible to study many of the reactions of the three isolates from equine milk and the one isolate from human milk, as a result of the failure to define the cultural conditions required for other than minimal growth.

Breakdown of carbohydrates

The basal medium used was Hiss's serum water prepared as described by Cruickshank (1960). One part of sterile bovine serum (Oxoid) was mixed with 3 parts of distilled water, and 20 ml of a 0.2% (w/v) solution of phenol red (see below) was added to each 400 ml of medium. The medium was adjusted to pH 7.6, distributed in 2.5 ml amounts in screw-capped ½ oz. (bijou) bottles and sterilized by steaming for 20 minutes on 3 successive days. The appropriate sterile carbohydrate solution (10% w/v) was added aseptically to the sterile basal medium

to give a final concentration of 1%. The stock solutions of carbohydrates and polyhydric alcohols etc. were sterilized by filtration through Carlson-Ford asbestos filters, Grade EKS (Carlson-Ford Sales Ltd., Ashton-under-Lyne, Lancs).

Phenol red solution

Phenol red (1.0 g) was dissolved in 28.4 ml of 0.1N sodium hydroxide with very gentle heating. Distilled water was added to approximately 400 ml then 28.4 ml of 0.1N hydrochloric acid were added. The solution was made up to 500 ml with distilled water and filtered before use.

The substrates used were:

Monosaccharide pentoses - arabinose, xylose.

Monosaccharide hexoses - fructose, galactose, glucose, mannose.

Disaccharide hexoses - lactose, maltose, sucrose.

Trisaccharide - raffinose.

Trihydric alcohol - glycerol.

Pentahydric alcohol - adonitol.

Hexahydric alcohols - dulcitol, mannitol, sorbitol.

Glucoside - salicin.

Hydroxypolymethylene - inositol.

After inoculation the screw caps were left slightly loose for 24 hours and then tightly closed, since Marcus & Greaves (1950) and Hayward (1957) noted the development of positive results in tightly closed bottles and tubes although loosely closed bottles and tubes exhibited no such reaction. The cultures were incubated at 37 °C or

30°C and examined daily for 6 days and then frequently for up to 8 weeks. The approximate pH of cultures was determined indirectly by visual comparison with control Hiss's serum waters adjusted in the pH range 4.8 - 8.0 as indicated by a Pye pH meter (W.G. Pye & Co. Ltd., Cambridge). Controls consisted of each strain inoculated into Hiss's serum water without fermentable substrate, and a full set of uninoculated media incubated for 5 weeks at 37°C.

Hydrolysis of starch

The basal medium, Hiss's serum water (see above) was distributed in 2.5 ml amounts in screw-capped ½ oz (bijou) bottles and sterilized by steaming for 20 minutes on each of 3 successive days. To each bottle of medium was added aseptically 0.5 ml of a sterile 5% (w/v) solution of soluble starch. The inoculated starch serum waters were incubated at 37°C (or 30°C) and examined for the production of acid daily for 6 days and frequently for up to 8 weeks.

In addition plates of starch agar were inoculated and incubated at 37°C (or 30°C) for 2, 5 and 9 days and examined for the hydrolysis of starch by flooding the plates with iodine solution. The plates were prepared by overlaying 10 ml of Tryptose Blood Agar Base (Oxoid) with 5 ml of starch agar (consisting of Tryptose Blood Agar Base with the addition of 1% starch).

Indole test

The medium, casitone tryptose serum broth, consisting of Bacto-Casitone (Difco), 5.0 g; tryptose (Oxoid), 5.0 g; Lab-Lemco beef extract (Oxoid), 3.0 g; sodium chloride, 5.0 g; distilled water, 1

litre; pH 7.2; was distributed in 25 ml amounts in 100 ml Erlenmeyer flasks to present a large surface area to the air since indole is best formed under aerobic conditions (Wilson and Miles, 1955). Sterilization was by autoclaving at 121°C for 15 minutes. Sterile bovine serum (Oxoid) was added aseptically after sterilization to a final concentration of 4% (v/v). After incubation for 5 and 10 days at 37°C the cultures were tested for the production of indole using Kovacs's indole test reagent (Report, 1958). A positive control, using Bacillus cereus, and a negative (uninoculated) control were also incubated and examined.

Production of hydrogen sulphide

The cultures were grown in casitone tryptose serum broth (see above) in 25 ml amounts in 100 ml Erlenmeyer flasks, and in 10 ml amounts in 6 x ⅝ inch test-tubes, with sterile lead acetate paper (Clarke, 1953) inserted into the neck of each flask or tube before incubation. Cultures were incubated for 21 days at 37°C and examined frequently. Hydrogen sulphide was indicated by blackening of the lead acetate paper. Negative reactors at the end of this period were confirmed by adding hydrochloric acid to each culture to liberate any dissolved sulphide, as described by Skerman (1959). Positive controls, using Proteus and Bacillus cereus, and a negative (uninoculated) control were also incubated and examined.

Production of ammonia from peptone or serum

The organisms were grown in casitone tryptose serum broth (see above) in 25 ml amounts in 100 ml Erlenmeyer flasks and in 10 ml

amounts in 6 x ⅝ test tubes. After incubation for 5, 10 and 18 days at 37°C two drops of culture medium were removed and mixed on a porcelain tile with 1 drop of Nessler's reagent. The production of ammonia was indicated by the rapid development of an orange or brick red colour. Positive controls, using Proteus and Bacillus cereus and a negative (uninoculated) control were set up and examined.

Reduction of nitrate

Nitrate serum broth consisting of: peptone (Oxoid), 10.0 g; sodium chloride, 5.0 g; potassium nitrate (analytical reagent grade), 1.0 g; distilled water, 1 litre; pH 7.4; was dispensed in test tubes with inverted Durham tubes, and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, sterile bovine serum was added aseptically, to a final concentration of 4% (v/v). After inoculation the cultures were incubated at 37°C for 12 and 18 days and the presence of nitrite was detected by the addition of Griess-Ilosvay's reagents, reduction of nitrate to nitrogen being detected by means of the Durham tubes. Negative reactors were confirmed by the further addition of zinc dust to reduce any nitrate present (Wilson & Miles, 1955). A positive control, using Pseudomonas, and a negative (uninoculated) control were also incubated and examined.

Urease production

The method of Christensen (1946) was employed, the medium consisting of Urea Broth Base (Oxoid) dispensed in 5 ml amounts in 6 x ⅝ test-tubes. After sterilization by autoclaving at 121°C for 15 minutes and cooling, 0.2 ml of sterile bovine serum and 0.25 ml of a sterile 40% solution of urea were aseptically added to each tube. Each strain was inoculated

into a tube of urea broth and a control tube of the basal medium containing serum but no urea. The inoculated tubes were incubated at 37°C for up to 22 days with frequent examination for positive reactors, which were indicated by a change in colour of the urea broth from straw to pink, with no similar change occurring in the control tube. A positive control, using Proteus, and a negative (uninoculated) control were also incubated and examined.

Liquefaction of gelatin

The liquefaction of gelatin was tested for by the use of both nutrient gelatin and gelatin-charcoal discs.

(a) Nutrient gelatin

The medium consisted of: Lab-Lemco beef extract (Oxoid), 10.0 g; peptone (Oxoid) 10.0 g; gelatin, 10.0 g; sodium chloride, 5.0 g; distilled water, 1 litre; pH 7.2. Sterilization was by steaming for 20 minutes on each of 3 consecutive days. After sterilization, sterile bovine serum was added aseptically to a final concentration of 3% (v/v). The medium was stab-inoculated and incubated at 37°C, liquefaction of gelatin being detected after incubation by cooling the tubes in iced water. In addition a further two sets of inoculated nutrient serum gelatins were incubated at 25°C, one set as stab cultures and the other set prepared as slants and surface streaked. Positive and negative controls were set up, using Pseudomonas, Proteus and Bacillus cereus as the positive controls.

(b) Charcoal-gelatin discs

In this method for detecting the liquefaction of gelatin, first described by Kohn (1953), discs of formalin-denatured gelatin containing

finely powdered charcoal are added to the nutrient medium in which the organisms are to be grown. The denatured gelatin will not melt when kept at 37°C for normal incubation periods, but it is still capable of being liquefied as a result of enzyme activity.

The basal medium consisted of Nutrient Broth No.2 (Oxoid) distributed in 3 ml amounts in 4 x ½ inch test tubes and sterilized by autoclaving at 121°C for 15 minutes. Sterile bovine serum (Oxoid) was added aseptically to a final concentration of 5% (v/v). One sterile Charcoal-Gelatin Disc (Oxoid) was added aseptically to each tube. The tubes were heavily inoculated, incubated at 37°C and examined after 8 hours, 24 hours and daily for up to 5 days.

Hydrolysis of casein

Layer plates were used, the base layer consisting of Tryptose Blood Agar Base (Oxoid) + 5% (v/v) of bovine serum, and the overlayer consisting of 5 ml of a mixture of 2 parts of 1½-strength Tryptose Blood Agar Base and 1 part of sterile reconstituted skim milk (Oxoid). Inoculated plates were incubated at 37°C for up to 9 days. Clear zones which developed during incubation were regarded as presumptive evidence of casein hydrolysis. In such cases confirmation was obtained by flooding the plates with mercuric chloride solution.

Liquefaction of coagulated serum

Loeffler's Serum Slopes (Oxoid prepared medium) in screw-capped ¼ oz (bijou) bottles were used. After inoculation, the bottles were incubated at 37°C, with the screw caps slightly loosened, for 7 days. Proteolysis was detected by the production of colonies surrounded by

craters of liquefied medium. Liquefaction was preceded by the development of translucency of the coagulated serum immediately surrounding the colonies.

Reaction in litmus skim milk

The medium consisted of Skim Milk Powder (Oxoid) reconstituted with distilled water to which was added sufficient 4% litmus solution (British Drug Houses Ltd.) to give a pale mauve colour. It was dispensed in 10 ml amounts in screw-capped 1 oz bottles and sterilized by autoclaving at 121°C for 5 minutes followed by steaming for 30 minutes on each of the two following days. After inoculation incubation was carried out with the screw caps slightly loosened, for 8 weeks at 37°C.

Egg-yolk agar

The basal medium was Tryptose Blood Agar Base (Oxoid) to which sodium chloride had been added, before sterilization to raise the final salt concentration to 1% (w/v). Ten ml of sterile Concentrated Egg Yolk Emulsion (Oxoid) were mixed with each 100 ml of molten sterile basal medium cooled to 50°C, and plates poured. After inoculation the plates were incubated at 37°C for 5 days.

Egg-yolk broth

The basal medium consisted of Nutrient Broth No.2 (Oxoid) to which a further 1% (w/v) of sodium chloride had been added before sterilization. It was dispensed in 5 ml amounts in 6 x $\frac{5}{8}$ inch test tubes and sterilized by autoclaving for 20 minutes at 121°C. After sterilization 0.25 ml of sterile Concentrated Egg Yolk Emulsion (Oxoid)

was added aseptically to each tube. The inoculated tubes were incubated at 37°C for three weeks, and were examined daily for the first three days and frequently thereafter.

Catalase production

The cultures were grown on egg-yolk agar plates for 3 days at 37°C. After incubation 1 ml of 10 vols hydrogen peroxide solution was poured over the surface of each plate, catalase production being indicated by effervescence in the hydrogen peroxide solution over the bacterial growth.

Tributyrin agar

Tributyrin Agar (Oxoid) was used with and without the addition of 5% (v/v) of sterile bovine serum (Oxoid). The inoculated plates were incubated at 37°C, and were examined for clearing daily for 5 days.

Tween agar (Sierra, 1957)

The basal medium consisted of Tryptose Blood Agar Base (Oxoid) to which was added sterile Tween 20 (polyoxyethylene sorbitan monolaurate), Tween 40 (polyoxyethylene sorbitan monopalmitate), Tween 60 (polyoxyethylene sorbitan monostearate), or Tween 80 (polyoxyethylene sorbitan mono-oleate), to a final concentration of 0.5% (v/v). The Tween was added to the molten basal medium at 50°C just before pouring the plates. After inoculation the plates were incubated at 37°C for 9 days, being examined daily for the production of zones of precipitation around the bacterial growth.

It was shown by the use of stock cultures known to give positive

reactions (Pseudomonas fluorescens, Alcaligenes viscolactis and Achromobacter lacticum) that the addition of calcium chloride to the basal medium, which was recommended by Sierra, was not necessary for well defined zones of precipitation.

Butter-fat agar (after Berry, 1944)

Butter-fat was prepared by melting unsalted butter at 50°C and separating the fat from the curds using a separating funnel. The butter-fat was distributed in 10 ml amounts in screw-capped 1 oz bottles and sterilized by autoclaving at 121°C for 15 minutes.

The medium was prepared by adding aseptically 5 ml of molten sterile butter-fat to 100 ml of molten sterile Tryptose Blood Agar Base (Oxoid) at 50°C, and emulsifying by vigorous hand-shaking immediately before pouring the plates. The inoculated plates were incubated at 37°C for 5 days. After incubation the amount of growth and any pigmentation was noted and the plates were then flooded with saturated copper sulphate solution and allowed to stand for 10 minutes. The reagent was then poured off and the plates washed gently in running water for one hour to remove the excess copper sulphate. Lipolysis of the butter-fat was indicated by bluish-green zones or the bacterial growth being coloured bluish-green, due to the formation of insoluble copper salts of the fatty acid set free on lipolysis.

Victoria blue butter-fat agar and Victoria blue margarine agar (Jones and Richards, 1952; Paton and Gibson, 1953).

Preparation of Victoria blue base Two grams of powdered Victoria blue (British Drug Houses Ltd.) were boiled in 200 ml of distilled water

until thoroughly dispersed. Sodium hydroxide solution (10% w/v) was added slowly with constant mixing until the coloration disappeared. The mixture was allowed to stand until the water-insoluble precipitate (the basic dye) had settled out. The precipitated basic dye was filtered off and washed with distilled water made slightly alkaline with ammonium hydroxide. The dye was finally dried at 30°C.

Preparation of butter-fat and margarine

Butter-fat was prepared as described for butter-fat agar (see above), and the margarine fat was similarly prepared.

Preparation of dye/fat mixture

One hundred grams of butter-fat or margarine were heated in a conical flask with 100 ml of water and a number of glass beads. When the mixture was boiling, basic Victoria blue powder was added slowly with constant mixing until the fat was saturated. This stage was indicated by the fat being deep red with particles of undissolved dye being present at the bottom of the flask. The mixture was boiled gently for a further 30 minutes. The fat was separated from the bulk of the water and filtered overnight at 37°C. The filtered fat was separated from any residual water, dispensed in screw-capped 1 oz bottles and sterilized by autoclaving at 121°C for 15 minutes.

Preparation of the complete medium

The basal medium consisted of Tryptose Blood Agar Base (Oxoid) in which the agar content had been increased to 2% (w/v) and the reaction of the medium adjusted to pH 7.8. This medium was dispensed in 20 ml amounts in screw-capped 1 oz bottles and sterilized by autoclaving at 121°C for 15 minutes. One ml of the basic dye/fat

mixture was added aseptically to each bottle of sterile molten medium at 45°C. The mixture was emulsified by vigorous hand-shaking for one minute and poured into a Petri dish.

The inoculated plates were incubated at 37°C for up to 9 days, and were examined daily for growth of the bacteria and for lipolytic activity as indicated by the formation of deep blue zones around the bacterial growth or by the bacterial growth being blue in colour.

Results

Breakdown of carbohydrates

The reactions of the isolates and of the type cultures in Hiss's serum water containing carbohydrates and similar substrates are summarized in Tables 4, 5 and 6. The reactions of the isolates of Corynebacterium bovis are shown in Table 4, and the reactions of the isolates not corresponding to C.bovis are given in Table 5. The typical reactions of these isolates are summarized in Table 6, with the reactions of the type cultures also being listed. It was observed that frequently reversals of pH occurred, the medium first turning acid and then after continued incubation becoming slowly alkaline. Many strains when inoculated into control serum waters containing no added substrate gave an alkaline reaction after prolonged incubation probably due to the production of ammonia from amino acids, and this could easily explain the reversals in pH which occurred. However, in a number of cases double reversals of pH were observed, in which the development of an acid reaction within about 7 days was followed by a change to alkalinity, followed after prolonged incubation by the development of an acid reaction once again. This type of reaction was given by at least one strain in each of the substrates xylose, mannitol, rhamnose, arabinose, dulcitol, raffinose, sorbitol, adonitol, galactose, maltose, fructose, mannose, glycerol. Some strains (e.g. strains 59 and 72) produced this type of reaction in a range of sugars. Since double reversals in pH were observed in such a wide range of substrates, including the monosaccharide fructose, no

Reaction	No. of strains showing indicated reaction in:																		
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Control (no substrate)
Production of acid (pH 5 or lower)																			
a) in 3 days	1	0	110	5	42	0	0	0	0	3	0	23	0	0	0	0	1	0	0
b) in 4-7 days	0	0	13	31	58	1	0	0	5	0	0	95	0	0	0	0	0	0	0
c) after >7 days	0	0	0	15	17	2	3	5	9	0	0	4	0	0	3	0	0	0	0
Production of weak acid (pH 5.5-7)	80	87	1	29	9	29	83	16	54	1	40	2	34	31	76	90	1	38	0
No change	14	8	0	40	0	66	9	92	40	103	49	1	55	57	15	4	5	42	71
Production of alkali	0	0	0	3	0	24	0	14	11	19	6	0	6	7	1	1	118	15	54
No. of strains tested	95	95	124	123	126	122	95	127	119	126	95	125	95	95	95	95	125	95	125

TABLE 4. THE BREAKDOWN OF CARBOHYDRATES IN HISS'S SERUM WATER BY C. BOVIS ISOLATES

Reaction	No. of strains* showing indicated reaction in:																		
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Control (no substrate)
Production of acid (pH 5 or lower) a) in 3 days			5N 1X	6N	6N	1N	3N	5N	2N	6N									
b) in 4-7 days			1N		1N	1N 1X	1N		2N	1X		1N							
c) after > 7 days					1X		3N 1M 1X		1X										
Production of weak acid (pH 5.5-7)	6N 3M 1X	7N 3M 1X	2N 1X	1N 4M	1N 3M 1X	2N 1M 1X	2M		3N 2M		3N 2M 1X	1X	2N 1X	2N 1M 1X	2N 1M 1X	3N 3M 1X		3N 1M 1X	
No change	1N 1M	1M	5M	1N 1M	2M	4N 1M	1M	2N 5M 2X	1N 3M	2N 4M 1X	4N 1M	2N 4M	4N 4M	4N 2M	5N 2M	4N 1M	1X	3N 3M	8N 5M
Production of alkali				2X		3M		1N	1X	1M	1M	5N 1M 1X	1N	1N 1M	1M		8N 5M 1X	1N	2X
No. of strains tested	7N 4M 1X	7N 4M 1X	8N 5M 2X	8N 5M 2X	8N 5M 2X	8N 5M 2X	7N 4M 1X	8N 5M 2X	8N 5M 2X	8N 5M 2X	7N 4M 1X	8N 5M 2X	7N 4M 1X	7N 4M 1X	7N 4M 1X	7N 4M 1X	8N 5M 2X	7N 4M 1X	8N 5M 2X

* No. followed by "N" for "nocardial" strains, "M" for Microbacterium isolates, "X" for other strains.

TABLE 5: THE BREAKDOWN OF CARBOHYDRATES IN HISS'S SERUM

WATER BY BOVINE MILK DIPHTHEROIDS OTHER THAN C.BOVIS

TABLE 6: SUMMARY OF THE BREAKDOWN OF CARBOHYDRATES IN HISS'S SERUM WATER

	ACID PRODUCTION FROM:																		
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Control (no substrate)
C.bovis isolates*	±	±	+	V(+)	+	V(-)	±	-	V(±)	-	V(-)	+	V(-)	V(-)	±	±	-	V(-)	-/OH
"Nocardial" isolates‡	±	±	±	±	±	V	±	±/-	V	±	V	-	V	V	V	V	-	V	-/OH
Microbacterium isolates*	±	±	-	±	V	-	V	-	V	-	V	-	-	V	V	±	-	-	-/OH
C.bovis NCTC 3224	±	±	±	-	±	±	±	-	±	-	±	-	±	±	±	±	-	±	-/OH
C.hofmannii NCTC 231	-	-	±	±	-	-	-	-	±	-	-	-	-	-	-	-	-	-	-/OH
C.flavidum NCTC 764	±	-	±	+	+	±	+	±	+	±	-	±	-	-	-	±	-	-	-/OH
C.ovis NCTC 3450	±	±	±	+	±	+	-	-	±	-	-	±	-	-	-	-	-	-	-/OH
D.diphtheriae NCTC 3985	±	-	±	+	±	+	+	-	+	±	-	+	-	-	-	±	-	±	-/OH
C.pyogenes NCTC 6448	-	-	±	+	+	±	-	±	+	+	-	+	-	-	-	-	±	-	-/OH
C.xerosis NCTC 7243	-	-	-	+	+	-	±	-	±	±	-	±	-	-	-	-	-	-	-/OH
9755	-	±	±	+	±	-	-	-	-	±	-	±	-	-	-	-	-	-	-/OH
C.renale NCTC 7448	-	-	+	-	+	+	±	-	±	-	-	-	-	-	-	-	-	-	-/OH
C.ulcerans NCTC 7910	-	±	+	±	±	±	±	-	±	±	-	±	-	-	-	±	-	-	-/OH
Microbacterium lacticum NCIB 8540 30°	NT	NT	+	+	+	±	NT	±	±	-	NT	±	NT	NT	NT	NT	±	NT	-/OH
37°			-	-	-	-		±	±	-		-					-		
NCIB 8541 30°	NT	NT	±	±	+	+	NT	-	±	±	NT	±	NT	NT	NT	NT	+	NT	-/OH
37°			±	±	±	±		-	±	-		-					±		
Equine diphtheroids	NT	NT	+	±	+	±	NT	±	±	±	NT	V	NT	NT	NT	NT	V	NT	-/OH

* + : final pH 5 or lower, in 80% or more of the strains
± : final pH 5.5-7.0, in 80% or more of the strains
- : no acid produced by 80% or more of the strains
V(+) : variable, no single reaction applying to 80-100% of the strains, but final pH of 5 or less predominant
V(±) : variable, no single reaction applying to 80-100% of the strains, but final pH of 5.5-7.0 predominant.
V(-) : variable, no single reaction applying to 80-100% of the strains, but absence of acid predominant.
‡ V : variable, no predominating reaction (too few cultures tested)
For all other cultures, +, final pH 5 or lower; ±, final pH 5.5-7.0; -, no acid produced.

TABLE 6: SUMMARY OF THE BREAKDOWN OF CARBOHYDRATES IN HISS'S SERUM WATER

satisfactory explanation for this phenomenon can be offered.

The reactions of C.bovis NCTC 3224 were similar to the typical reactions of the C.bovis isolates except that acid was not produced from glycerol by C.bovis NCTC 3224 (see Table 6). C.ulcerans NCTC 7910 differs from the typical C.bovis isolates in its reactions to arabinose, sucrose and mannitol.

Hydrolysis of starch

None of the C.bovis isolates was capable of growth on starch agar, and none gave a reaction in Hiss's serum water containing soluble starch. The "nocardial" isolates grew on starch agar and 3 of the 8 strains produced narrow zones of hydrolysis of the starch, the remaining 5 producing no reaction. None of these 8 strains gave a reaction in the starch serum water.

All 5 of the Microbacterium isolates grew moderately well on starch agar with wide zones of hydrolysis, but none produced acid in starch serum water.

The reactions of these and of the type cultures in starch agar and starch serum water are shown in Table 7.

Indole test

None of the isolates and type cultures produced indole from casitone tryptose serum broth during incubation at 37°C for 10 days.

Production of hydrogen sulphide

None of the C.bovis isolates or "nocardial" isolates produced hydrogen sulphide from casitone tryptose serum broth. Three of the 5 Microbacterium isolates produced slight blackening of the lead acetate paper after incubation for 5-10 days at 37°C. None of the 3 equine

	Starch Agar		Acid production in starch serum water
	Growth	Reaction	
C.bovis isolates	-	...	-
"Nocardial" isolates	+++	- or +	-
Microbacterium isolates	+	++	-
Equine diphtheroid (one strain tested)	NT	NT	-
Human diphtheroid	NT	NT	-
C.bovis NCTC 3224	-	...	-
C.hofmannii NCTC 231	+++	-	-
C.flavidum NCTC 764	+++	+	±
C.ovis NCTC 3450	++	+	-
C.diphtheriae NCTC 3985	+++	++	+ 8 days
C.pyogenes NCTC 6448	-	...	± 9 days, + 7 weeks
C.xerosis NCTC 7243	+++	+	-
NCTC 9755	+++	±	-
C.renale NCTC 7448	++	-	-
C.ulcerans NCTC 7910	+++	++	+ 2 days
Microbacterium lacticum NCIB 8540 tested at 30°C	+++	++	-
tested at 37°C	+++	++	-
M.lacticum NCIB 8541 tested at 30°C	+++	-	-
tested at 37°C	++	±	-

Note: Growth on starch agar: +++, very good growth; ++, good growth; +, moderate growth; -, no growth. Reaction on starch agar: ++, large zones of diastasis; +, moderate zones of diastasis; ±, slight hydrolysis of starch; -, no starch hydrolysis; ..., not applicable. Acid production in starch serum water: +, acid produced to a final pH of 5 or less (followed by incubation required for this amount of acid production); ±, acid produced to a final pH of 5.5 to 7.0: -, no change.

TABLE 7: HYDROLYSIS OF STARCH

diphtheroids produced hydrogen sulphide.

C.hofmannii NCTC 231, C.bovis NCTC 3224, C.ovis NCTC 3450, C.pyogenes NCTC 6448, C.xerosis NCTC 7243, C.renale NCTC 7448 and Microbacterium lacticum NCIB 8540 and 8541 produced no detectable hydrogen sulphide.

C.ulcerans NCTC 7910 produced sufficient hydrogen sulphide to cause considerable blackening of the lead acetate paper within 5 days in both flask and test-tube cultures. C.flavidum NCTC 764 gave a negative reaction in the flask culture, but produced some blackening of the lead acetate paper in the case of the test tube culture. The reaction of C.flavidum would thus appear to support the view of Skerman (1959) that hydrogen sulphide production is enhanced when cultures are grown under conditions of severe oxygen limitation. However, both C.diphtheriae NCTC 3985 and C.xerosis NCTC 9755 produced hydrogen sulphide when grown in shallow cultures in Erlenmeyer flasks, but not when grown in test-tube cultures, in the case of C.xerosis considerable blackening of the lead acetate paper occurring within 5 days.

Production of ammonia

Cultures of all the isolates from bovine milk (grown in casitone tryptose serum broth) gave a colour reaction with Nessler's reagent slightly more orange than the colour reaction obtained with the uninoculated control broth, indicating slight ammonia production. C.ulcerans NCTC 7910 and C.pyogenes NCTC 6448 gave a strong reaction, with a deep brick-red colour developing when Nessler's reagent was added, all other type cultures producing a colour reaction slightly

more orange than that obtained with the uninoculated medium. This confirms the slow development of alkalinity in Hiss's serum water containing no carbohydrate substrate.

Reduction of nitrate

Nitrate was reduced to nitrite by two of the C.bovis isolates, one of the two corynebacterial strains not corresponding to C.bovis, 6 of the 8 "nocardial" isolates, two of the three equine diphtheroids, and the type cultures of C.hofmannii, C.flavidum, C.diphtheriae C.xerosis NCTC 9755, and Microbacterium lacticum (M.lacticum NCIB 8540 being positive at 30°C, negative at 37°C, M.lacticum 8541 negative at 30°C, positive at 37°C).

Nitrate was not reduced by 125 of the 127 C.bovis isolates, the isolates of Microbacterium or the type cultures of C.bovis, C.pyogenes C.xerosis NCTC 7243, C.renale and C.ovis.

Urease production

Urea was hydrolysed by most of the C.bovis isolates in 24-48 hours 123 gave positive results within 6 days; two more strains gave positive results in 7-11 days; only two of the 127 strains continued to give negative results after prolonged incubation. Urea was also hydrolysed by all of the isolates of Microbacterium, one of the "nocardial" isolates, and within 48 hours by the type cultures of C.hofmannii, C.renale, C.ovis and C.ulcerans. None of the strains gave a false positive reaction in the Christensen's medium without urea. The three isolates of corynebacteria from equine milk were negative reactors. The type cultures of C.bovis, C.flavidum,

C.diphtheriae, C.pyogenes, C.xerosis and Microbacterium lacticum did not hydrolyse urea.

Liquefaction of gelatin

Only C.pyogenes NCTC 6448 was able to liquefy the nutrient serum gelatin stab within 4 weeks at 25°C or 37°C. None of the cultures liquefied charcoal-gelatin discs within 48 hours at 37°C. Incubation of charcoal-gelatin discs for longer than 48 hours at 37°C caused the slow disintegration of the discs in the uninoculated controls. Nutrient serum gelatin slants provided a more sensitive indication of proteolysis, and slight liquefaction was evident when slant cultures were incubated at 25°C in the case of C.ulcerans NCTC 7910, the five Microbacterium isolates and the nocardial isolate strain No.39 (in addition to C.pyogenes NCTC 6448).

Hydrolysis of casein

The C.bovis isolates grew only sparsely on skim milk agar in regions of heavy inoculation, with no detectable casein hydrolysis. The Microbacterium isolates grew moderately well, and the "nocardial" isolates gave good growth on skim milk agar, with two of the "nocardial" isolates exhibiting narrow zones of casein hydrolysis.

The type cultures of C.hofmannii, C.ovis, C.renale and Microbacterium lacticum grew well on skim-milk agar, with all except C.hofmannii producing wide zones of hydrolysis.

The type cultures of C.bovis and C.pyogenes grew only sparsely on skim milk agar, but C.pyogenes produced large zones of caseinolysis.

Liquefaction of coagulated serum

Only C.pyogenes NCTC 6448 caused noticeable liquefaction of the

coagulated serum.

Reaction in litmus skim milk

Five of the 127 C.bovis isolates produced slight acidity in litmus skim milk, the remaining 122 strains producing no reaction due to an inability to multiply in the medium. Of the 5 "microbacterial" isolates, two produced an acid clot followed by reduction of the litmus, and the remaining three strains caused the medium slowly to become deeply alkaline. Seven of the eight "nocardial" isolates produced no reaction in litmus skim milk, the remaining strain causing the medium to become alkaline.

Of the type cultures, C.pyogenes and Microbacterium lacticum NCIB 8540 produced acid clotting of the litmus skim milk, the latter strain giving a reaction only at 30°C, and not at 37°C. C.hofmannii, C.ovis and M.lacticum NCIB 8541 produced no reaction. C.renale produced an alkaline reaction in the medium.

Reaction on egg-yolk agar and in egg-yolk broth

All the isolates from bovine milk gave profuse growth on egg-yolk agar and in egg-yolk broth although producing no reaction in the medium. One of the equine diphtheroids and the human diphtheroid were tested in these media: they gave very little growth and no lecithinase reaction. All but one of the type cultures grew well, the exception being C.pyogenes which gave very sparse growth in the absence of serum. Only C.ulcerans produced a reaction, producing opacity in egg-yolk broth, although giving no reaction in egg-yolk agar.

Tributyrin agar

Little growth of the C.bovis isolates was obtained on tributyrin

agar and then only in the region of the heaviest inoculum. Very slight clearing of the medium was sometimes associated with this growth. The lack of growth appeared to be due to inhibition of the organisms by the tributyrin since the addition of serum to the tributyrin agar did not significantly increase the amount of growth obtained. In a further study using a few typical isolates it was found that by the use of heavily inoculated single streaks, although the amount of growth obtained was not increased, the amount of clearing could be increased from being barely discernible to a quite obvious clearing of the medium under the streak. However, the full depth of the medium was not cleared (See Plate 1).

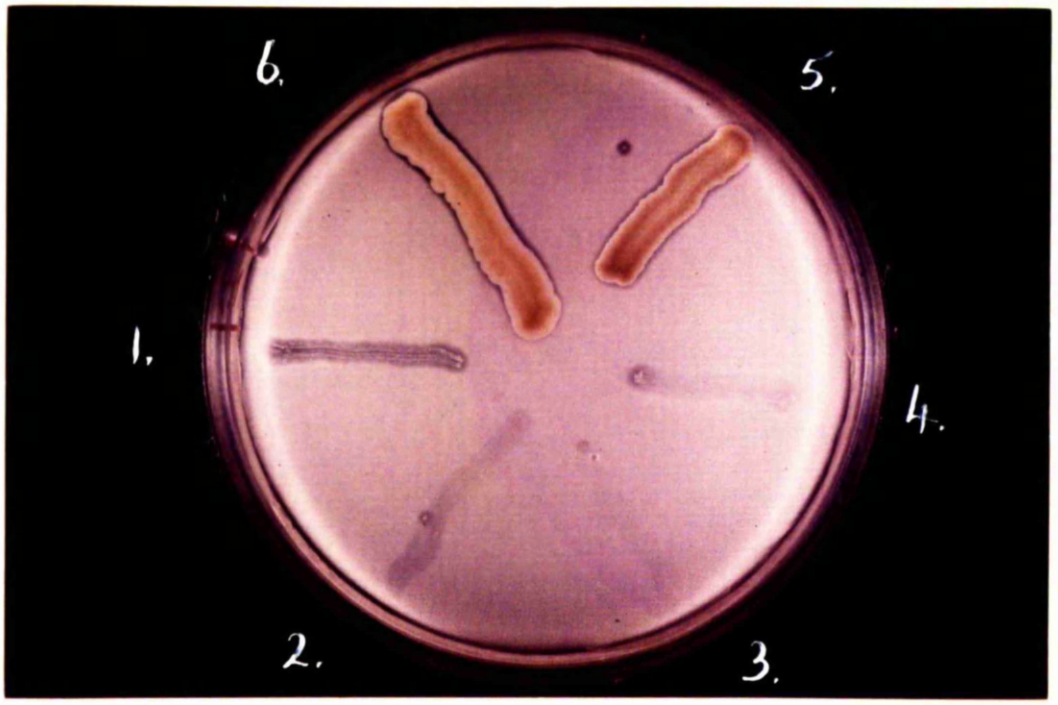
Six of the 8 "nocardial" isolates grew luxuriantly on tributyrin agar, producing a zone of clearing 2 mm wide; the remaining two strains grew only sparsely on tributyrin in the region of the heaviest inoculum.

The five Microbacterium isolates gave moderate growth, and clear zones 1-2 mm wide.

Of the type cultures, C.bovis, C.hofmannii, C.pyogenes and Microbacterium lacticum grew only sparsely on tributyrin agar, even with the addition of serum, C.bovis being the only one of these five strains to produce a detectable clearing of the tributyrin agar and then only in the region of the heaviest inoculum. Corynebacterium flavidum, C.xerosis and C.ulcerans grew luxuriantly on tributyrin agar without the need for the addition of serum, C.flavidum and both strains of C.xerosis producing zones of clearing. C.ovis and C.diphtheriae grew moderately well on tributyrin agar, the growth of

PLATE 1. GROWTH AND REACTION OF CORYNEBACTERIUM ON TRIBUTYRIN AGAR

Incubated for 3 days at 37°C



1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis strain 159
4. C.bovis NCTC 3224
5. C.xerosis NCTC 7243
6. C.xerosis NCTC 9755

C.diphtheriae being much enhanced (with very slight clearing of the medium) by the addition of serum.

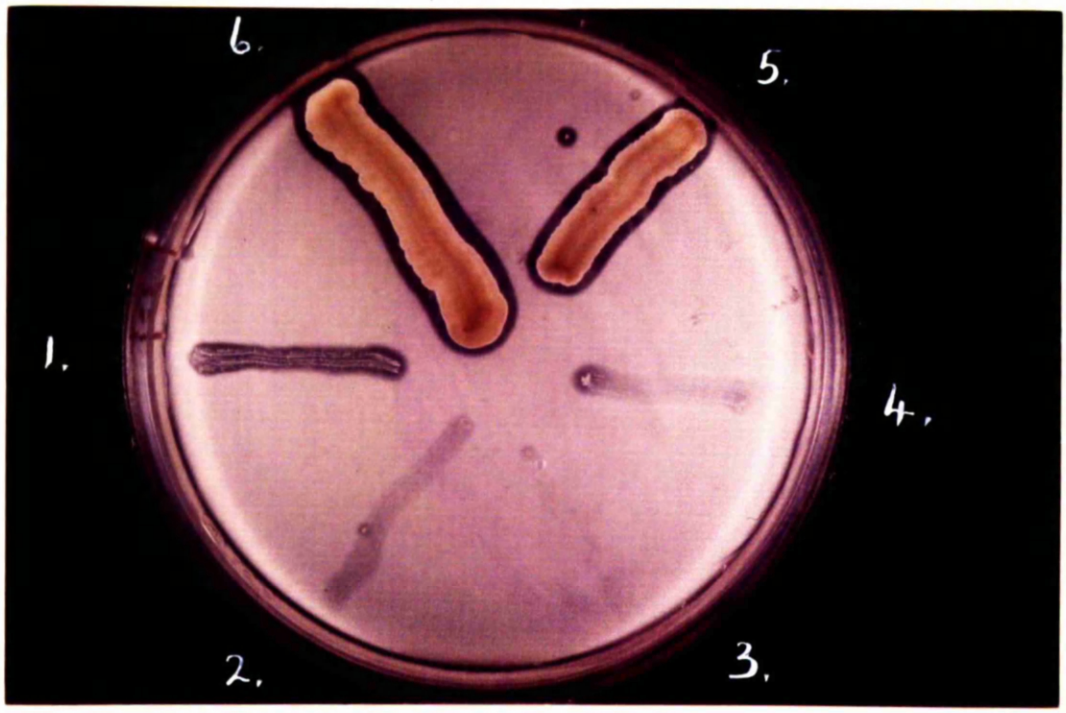
The amount of clearing of the tributyrin agar produced by C.bovis (both the type culture and typical isolates), C.flavidum and C.xerosis was increased greatly by further incubation of the plates at 20°C for 5 days after incubation at 37°C for 3 days (Plate 2), as compared with plates incubated at 37°C for the whole period of time. A similar effect was noted in the reaction of these cultures on Victoria blue butter-fat agar and the Tween agars (see below). Thus, although the optimum temperature for growth of these three organisms is 37°C, the rate of hydrolysis of tributyrin and butterfat would appear to be increased at lower temperatures.

Tween agar

The C.bovis isolates grew luxuriantly on Tween 20 agar and Tween 80 agar and produced broad zones of precipitation (up to 3 mm wide after 4 days at 37°C, and extending to 1 cm wide after 1 week). No growth was obtained on Tween 40 agar and very little was obtained on Tween 60 agar. Six of the eight nocardial isolates grew well on all four Tween agars, producing precipitation only on Tween 20 agar and Tween 40 agar; one strain grew luxuriantly on Tween 40 agar and Tween 60 agar with zones of precipitation being produced, but it failed to grow on Tween 20 agar and Tween 80 agar; the remaining strain grew well on Tween 20 agar and Tween 80 agar producing zones of precipitation but was unable to grow on Tween 40 agar and Tween 60 agar. All five Microbacterium strains grew well on all four media, with zones of

PLATE 2. GROWTH AND REACTION OF CORYNEBACTERIUM ON TRIBUTYRIN AGAR

Incubated for 3 days at 37°C, followed by 5 days at 20°C



1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis strain 159
4. C.bovis NCTC 3224
5. C.xerosis NCTC 7243
6. C.xerosis NCTC 9755

TABLE 8: GROWTH AND REACTION ON TWEEN AGARS

	Tween 20 agar		Tween 40 agar		Tween 60 agar		Tween 80 agar	
	Growth	Reaction	Growth	Reaction	Growth	Reaction	Growth	Reaction
C.bovis isolates	+++	++	-	...	(±)	-	+++	++
"Nocardial" isolates	+++	++	+++	++	+++	-	+++	-
Microbacterium isolates	+++	++	+++	++	+++	++	+++	++
C.bovis NCTC 3224	+++	++	(±)	-	(±)	-	+++	++
C.hofmannii NCTC 231	++	++	±	+	++	±	+++	-
C.flavidum NCTC 764	+++	++	++	+	+++	++	+++	++*
C.ovis NCTC 3450	+++	++	+	+	±	++	+++	±
D.diphtheriae NCTC 3985	+++	++	++	+	+++	-	+++	++*
C.pyogenes NCTC 6448	±	-	(±)	-	(±)	-	-	...
C.xerosis NCTC 7243	+++	++	(±)	-	(±)	-	+++	++
NCTC 9755	+++	++	+++	++	+++	++	+++	++
C.renale NCTC 7448	+++	++	+	++	++	-	+++	±
C.ulcerans NCTC 7910	+++	++*	++	+	+++	++	+++	++*
Microbacterium lacticum NCIB 8540 tested at 30°C	+++	-	+++	-	NT	NT	+++	-
tested at 37°C	-	...	±	-	±	-	(±)	-
NCIB 8541 tested at 30°C	+++	-	+++	-	NT	NT	+++	-
tested at 37°C	-	...	±	-	±	-	±	-

Note. Growth: +++, excellent; ++, good; +, moderate; ±, slight; (±), slight and only from a heavy inoculum; -, no growth. Reaction: ++, wide zones of heavy precipitation; +, wide zones of slight precipitation; ±, narrow zones of slight precipitation; -, no precipitation; ..., not applicable. NT, not tested.

* Slow development of zones of precipitation, particularly when kept 3 days at 37°C and then at 20°C.

TABLE 8: GROWTH AND REACTION ON TWEEN AGARS

precipitation.

The typical reactions of the isolates and of the type cultures are shown in Table 8. The growth and reactions of two typical C.bovis isolates, C.bovis NCTC 3224 and the type culture of C.ulcerans on Tween 20 agar and Tween 80 agar are shown in Plates 3 and 4.

Butter-fat agar

The C.bovis isolates (123 of the 127 strains were tested) all grew well on butter-fat agar and were pigmented white (37 strains), cream (37 strains) or even golden yellow (49 strains) (Plate 5). When the plates were treated with saturated copper sulphate solution 63 strains gave varying degrees of blue-green coloration indicative of copper salts of the fatty acids liberated by hydrolysis of the butter-fat, and 60 strains produced no reaction (Plate 6). The depth of pigmentation seemed to be correlated with lack of reaction as indicated by the copper sulphate treatment (Table 9).

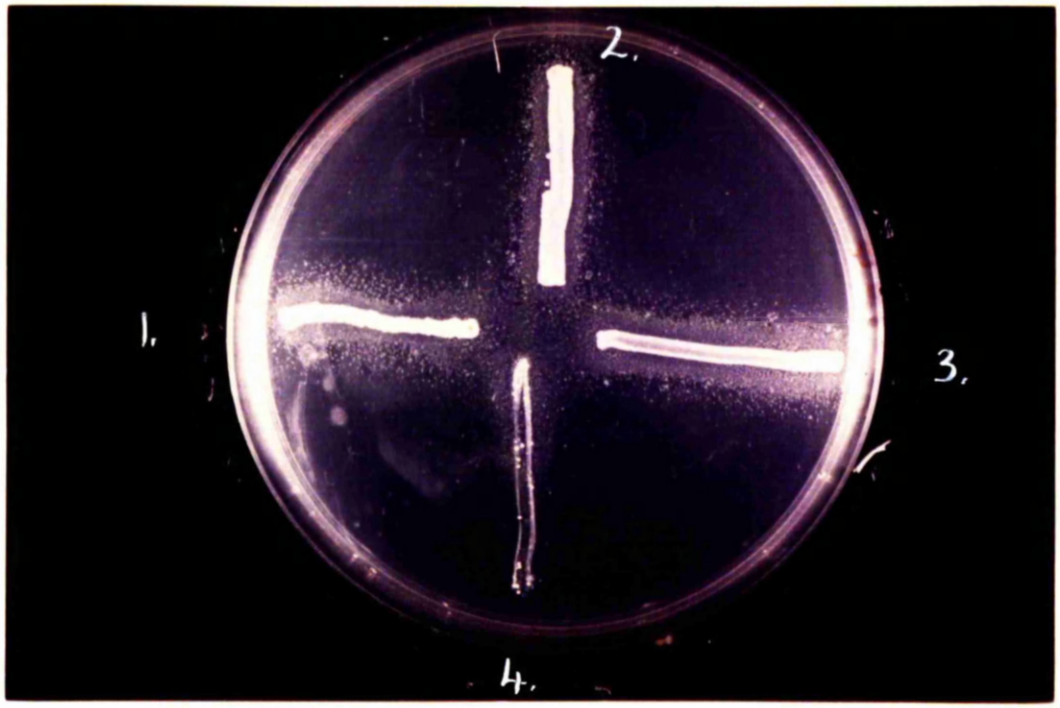
TABLE 9

PIGMENTATION ON, AND HYDROLYSIS OF, BUTTER-FAT AGAR BY C.BOVIS ISOLATES

Pigmentation	Number of strains giving reaction indicated ++	+	-	Total number of strains
Yellow	0	9	40	49
Cream	13	7	17	37
White	30	4	3	37
Total number of strains	43	20	60	

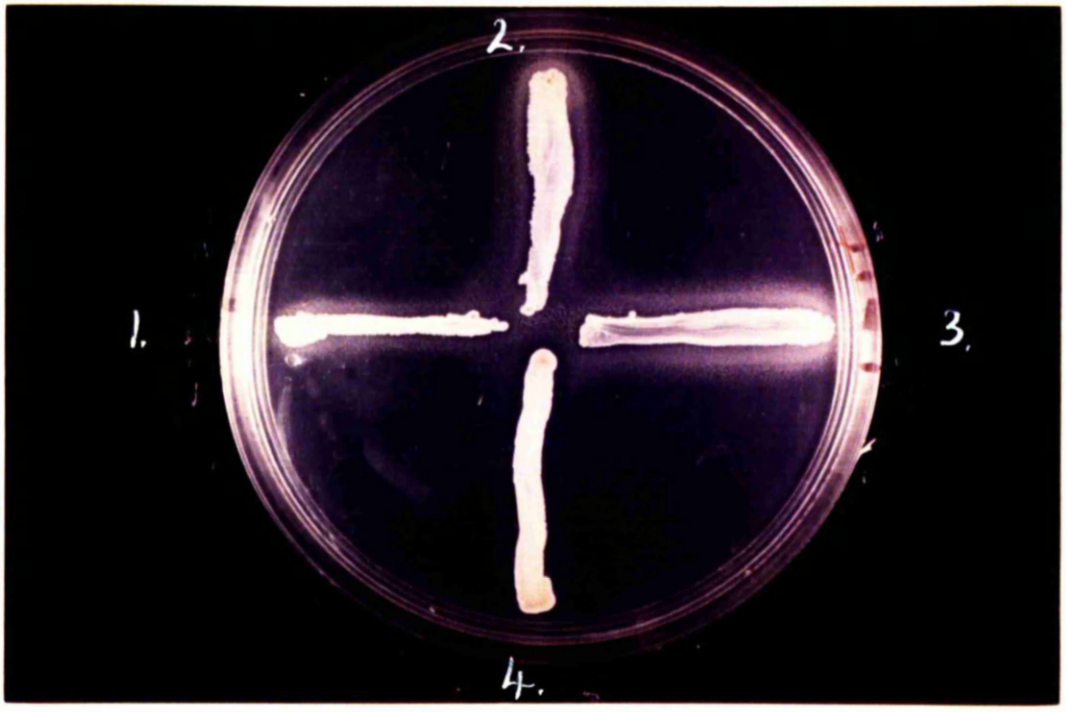
Note: ++, bacterial growth stained deeply when treated with $CuSO_4$ solution; +, bacterial growth lightly stained when treated with $CuSO_4$ solution; -, no reaction with $CuSO_4$ solution.

PLATE 3. GROWTH AND REACTION OF CORYNEBACTERIUM ON TWEEN 20 AGAR



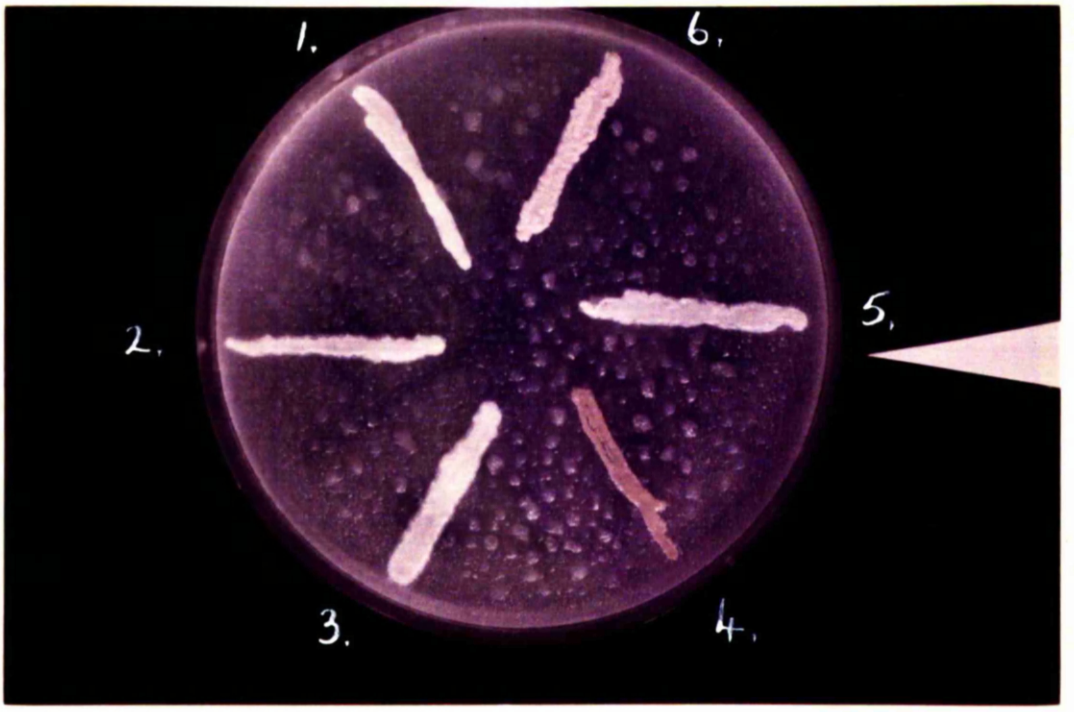
1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis NCTC 3224
4. C.ulcerans NCTC 7910

PLATE 4. GROWTH AND REACTION OF CORYNEBACTERIUM ON TWEEN 80 AGAR



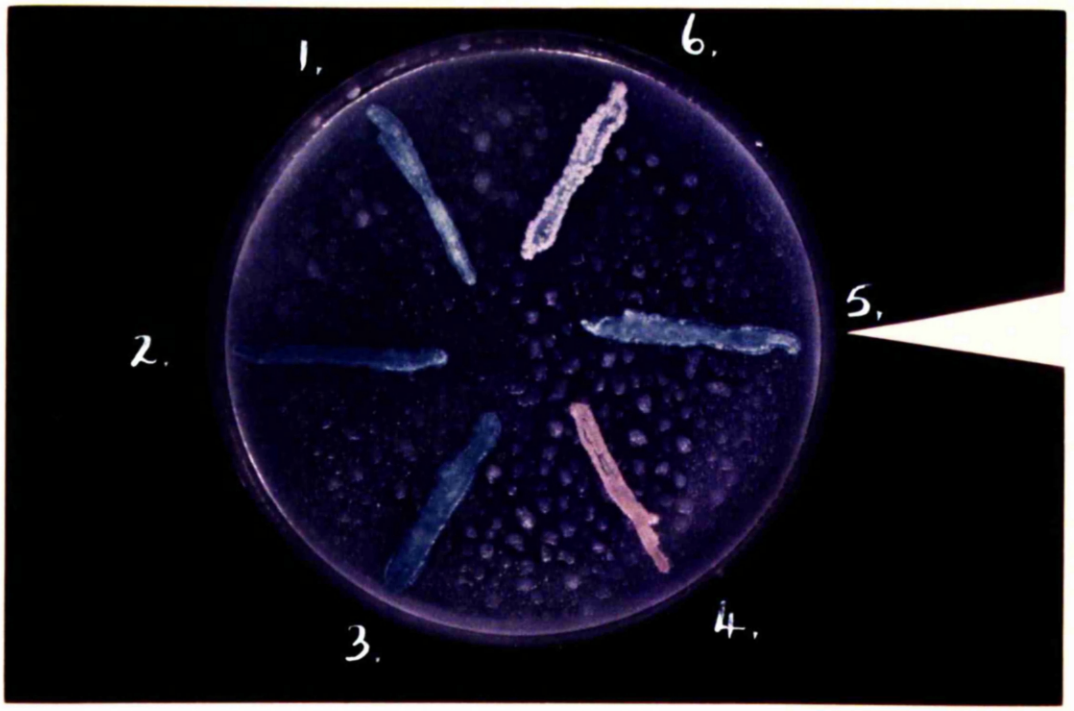
1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis NCTC 3224
4. C.ulcerans NCTC 7910

PLATE 5. GROWTH OF CORYNEBACTERIUM ON BUTTER-FAT AGAR



1. C.bovis strain 124
2. C.bovis strain 159
3. C.bovis strain 145
4. C.bovis strain 147
5. C.xerosis NCTC 7243
6. C.diphtheriae NCTC 3985

PLATE 6. REACTION OF CORYNEBACTERIUM ON BUTTER-FAT AGAR USING COPPER SULPHATE SOLUTION



1. C.bovis strain 124
2. C.bovis strain 159
3. C.bovis strain 145
4. C.bovis strain 147
5. C.xerosis NCTC 7243
6. C.diphtheriae NCTC 3985

The "nocardial" strains grew poorly on butter-fat agar, and produced no reaction as determined by copper sulphate solution. The Microbacterium strains similarly produced no reaction but gave abundant growth on the medium.

All the type cultures of Corynebacterium tested (C.pyogenes and C.ovis were not tested) gave good growth on butter-fat agar. In the case of C.hofmannii, C.bovis and C.xerosis NCTC 7243 the bacterial growth was deeply stained by treatment with copper sulphate, whereas C.flavidum, C.diphtheriae, C.renale, C.ulcerans and C.xerosis NCTC 9755 gave no reaction. The two type cultures of Microbacterium lacticum grew very poorly on butter-fat agar, with no reaction when treated with copper sulphate solution.

Growth and reactions on Victoria blue margarine agar and Victoria blue butter-fat agar

The amount of growth of the C.bovis isolates obtained on Victoria blue margarine agar was extremely small and a very heavy inoculum was required to obtain moderate growth. The bacterial growth was usually coloured blue, suggesting hydrolysis of the fat, although no blue zones were produced. When serum was added to this medium, the amount of growth obtained was comparable with that obtained on the usual serum agar, but because of the addition of the serum, colour changes occurring in the medium were not indicative of lipolysis alone, but could be caused for example by acid production from the carbohydrates present in the medium. The "nocardial" isolates and Microbacterium isolates grew somewhat better on Victoria blue margarine agar, although not growing as abundantly as on serum

ager or egg-yolk agar, for example. The growth of the "nocardial" isolates was coloured blue, suggesting lipolysis of the margarine, although no blue zones were produced around the bacterial growth, whereas the isolates of Microbacterium exhibited no blue coloration.

The type cultures of C.hofmannii, C.bovis, C.xerosis NCTC 7243 and C.renale required a heavy inoculum to obtain only very slight growth, but the bacterial growth was coloured blue (no zonation occurring). These cultures grew better when serum was incorporated in the medium. The type cultures of C.pyogenes and C.ovis were unable to grow at all on this medium, even when serum was incorporated. C.xerosis NCTC 9755 grew moderately well on the medium without the need for the addition of serum, but no colour reaction was produced. Both type cultures of Microbacterium lacticum grew well on Victoria blue margarine agar, with no colour reaction.

The use of Victoria blue butter-fat agar proved difficult as there was a tendency for the medium to turn blue spontaneously. This tendency is the reason for Victoria blue margarine agar being preferred by many workers. In addition to using various types of butter, a number of modifications in the medium were tried involving alterations in the pH of the basal medium, alterations in the amount of Victoria blue incorporated and in its method of incorporation. Eventually a suitable medium was prepared according to the standard method already noted, using as the source of butter-fat an unsalted Normandy butter. This medium was blue when the plates were first prepared but after storage at room temperature for one week (with the plates being stored in sealed polythene bags to prevent excessive desiccation) the medium

became pinkish-mauve in colour. If inoculated at this stage, lipolysis could be detected by the usual development of a blue coloration of the bacterial growth, and/or the development of blue zones around the colonies.

Thirty of the 87 C.bovis isolates tested grew only sparsely on Victoria blue butter-fat agar, but all of these strains were coloured slightly blue and four of the strains produced very narrow blue zones in the medium around the bacterial growth. Fifty strains grew moderately well on this medium, 10 strains producing very narrow blue zones in the medium in addition to the bacterial growth being coloured deep blue, seven strains giving growth that was deep blue but producing no zonation (Plate 7). Intensification of the colour reaction was noted when the plates were incubated for 3 days at 37°C followed by incubation for 5 days at 20°C (Plate 8). The growths of the remaining 32 strains were coloured slightly blue. Seven strains were incapable of growth on this medium.

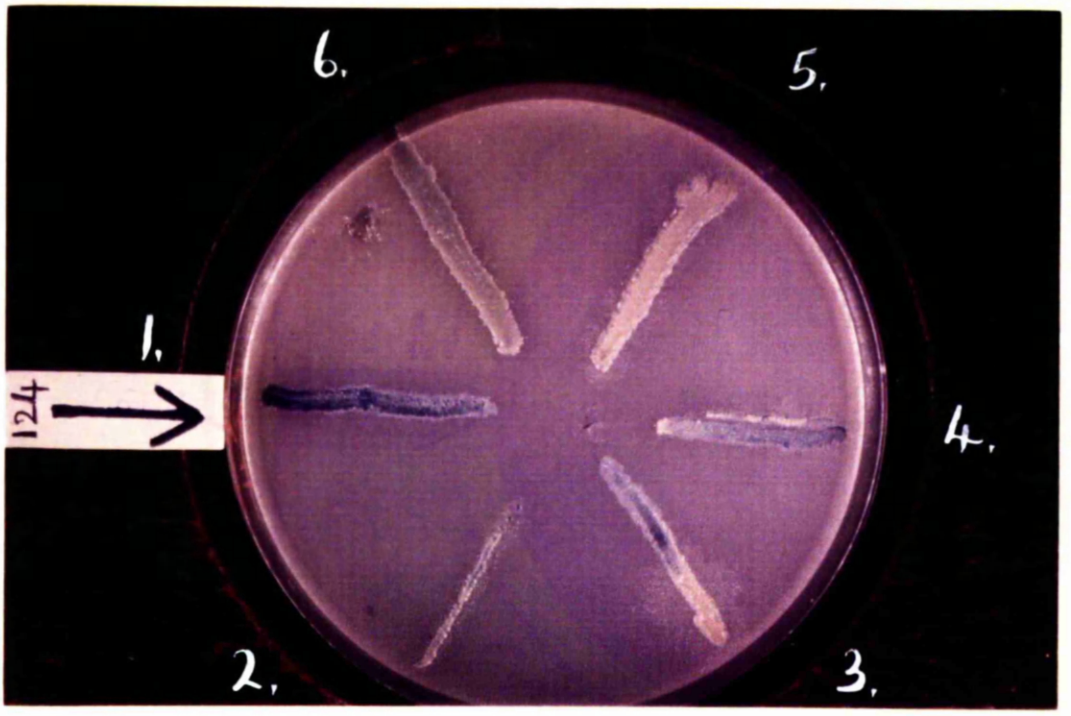
All eight "nocardial" strains grew moderately well on Victoria blue butter-fat agar, six producing growth that was coloured slightly blue, the remaining two strains producing no reaction.

The five isolates of Microbacterium grew moderately well on Victoria blue butter-fat agar but with no indication of lipolysis.

The two type cultures of C.xerosis grew extremely well on Victoria blue butter-fat agar, C.xerosis NCTC 7243 producing no reaction, and the growth of C.xerosis NCTC 9755 being coloured slightly blue. The type cultures of C.bovis and C.renale gave

PLATE 7. GROWTH AND REACTIONS OF CORYNEBACTERIUM ON VICTORIA BLUE BUTTER-FAT AGAR

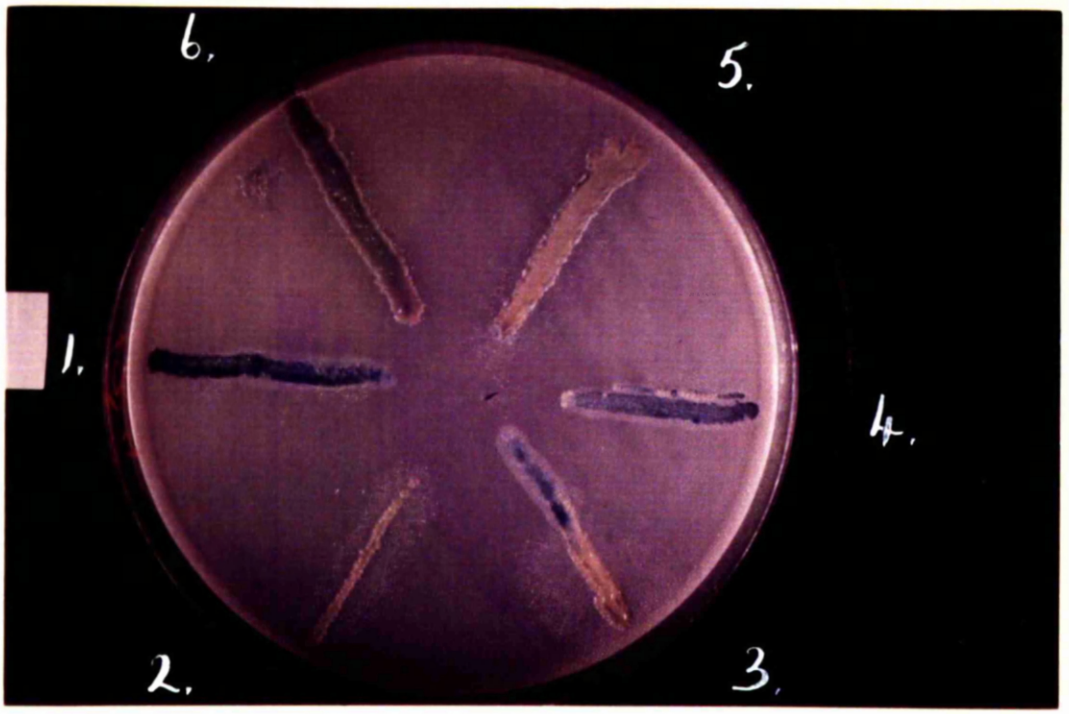
Incubated for 3 days at 37°C



1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis strain 159
4. C.bovis NCTC 3224
5. C.xerosis NCTC 7243
6. C.xerosis NCTC 9755

PLATE 8. GROWTH AND REACTIONS OF CORYNEBACTERIUM ON VICTORIA BLUE BUTTER-FAT AGAR

Incubated for 3 days at 37°C, followed by 5 days at 20°C.



1. C.bovis strain 124
2. C.bovis strain 147
3. C.bovis strain 159
4. C.bovis NCTC 3224
5. C.xerosis NCTC 7243
6. C.xerosis NCTC 9755

moderately good growth on this medium, the growth of C.bovis being coloured slightly blue, and C.renale producing no reaction. C.ulcerans NCTC 7910 grew only sparsely and showed no evidence of lipolysis. The type cultures of C.hofmannii and Microbacterium lacticum were unable to grow on Victoria blue butter-fat agar.

Discussion and Conclusions

The isolates of C.bovis produced positive reactions in a number of biochemical test media, in contrast to the usual descriptions of the biochemical inactivity of this organism. For example, the isolates produced sufficient acid from glucose, fructose and glycerol in Hiss's serum water to cause coagulation of the medium (Table 4). Acid production was most rapid when fructose was the substrate with the majority of strains producing coagulation of the medium within 3 days when incubated at 37°C. Acid was produced by more than 80% of the strains from arabinose, xylose, rhamnose, mannitol and sorbitol. Many of the isolates also produced acid from galactose and maltose. The type culture, C.bovis NCTC 3224, differed from the isolates in its inability to produce acid from glycerol, but otherwise the reactions in Hiss's serum water were very similar. Thus C.bovis, in common with other animal-parasitic corynebacteria (with the exception of C.hofmannii), is quite active against carbohydrates provided that Hiss's serum water and not peptone water is used as the basal medium.

Although a serum-containing medium has been regularly employed in the past in the examination of the biochemical reactions of C.diphtheriae, such use appears not to have been generally extended to studies of C.bovis. Evans in 1916 noted the enhancement of growth of Bacillus abortus var. lipolyticus (or Bacterium lipolyticum, now regarded as being synonymous with C.bovis) by the addition of serum to the medium. Nevertheless her finding that this organism produced no acid from carbohydrates suggests that for the determination of biochemical reactions she used basal media containing no serum, since in the present

work it was found that the type culture C.bovis NCTC 3224, which is one of Evans's strains of Bacterium lipolyticum, produced acid from a number of carbohydrates (See Table 6). Black (1941) and Breiullaud and Michel (1962) have also reported C.bovis to be inactive against carbohydrates. The "Type A udder corynebacteria" of Bendixen (1933) and the strains of C.pseudodiphthericum type II of Ochi and Zaizen (1940) were reported by these workers to be inactive against carbohydrates. Both of these bacterial types appear to have been identical with C.bovis in their cultural and gross morphological characteristics as well as in their natural habitat; indeed, Ochi considers (Katsube, 1964, personal communication) C.bovis to be one member of the more widely defined species C.pseudodiphthericum Type II.

All the reports or descriptions of C.bovis in which it is stated to be incapable of producing acid from carbohydrates are characterized by either a lack of information concerning the basal medium used, or by the use of basal media such as nutrient broth or peptone water with no supplementation by the addition of serum or other growth-promoting substances. Unfortunately reports of this type appear to have been the chief sources for such works of reference as those of Breed and colleagues (1957) and Cowan & Steel (1965). There are two main types of biochemical tests: those which require growth of the organisms before a reaction can be detected, and those carried out on resting, non-growing or non-reproducing cells. Most of the biochemical tests routinely employed are of the first type and such tests obviously necessitate the use of basal media capable of supporting the growth of the organisms

being examined. Nevertheless in the past the biochemical characteristics of C.bovis would appear to have been determined with inadequate basal media. The results given here, together with the published reports (Cobb and Walley, 1962; Cobb 1963, 1966) of investigations on C.bovis which were carried out simultaneously with the present work, present overwhelming evidence of the carbohydrate-dissimilating powers of C.bovis when suitable media are employed. This is adequately confirmed by the finding of Jayne-Williams and Skerman (1966) that resting cells of C.bovis are capable of producing acid from a number of carbohydrates when the cells are suspended in substrate solution containing a pH indicator and incubated for up to 24 hours at 37°C.

Another biochemical test in which C.bovis gave rapid and unequivocal results was that for the hydrolysis of urea. Only two of the 127 isolates examined failed to hydrolyse urea. In this respect they differed from Evans's strain (NCTC 3224) which is urease negative.

The reactions of Corynebacterium bovis on the various media for detecting lipolysis were in some cases difficult to interpret. The weakness of the reaction produced by C.bovis on tributyrin agar that was noted by Black (1941) appears to be due to the inhibitory effect of tributyrin since the addition of serum caused little if any increase in the amount of growth obtained. Use of very heavy inocula increased slightly the amount of clearing obtained. This suggests that C.bovis can produce a lipase in a suitable environment. It should be noted however that Sierra (1964) reported clearing of tributyrin agar by a proteolytic enzyme. Although C.bovis has not been demonstrated to produce a

proteolytic enzyme, the use of tributyrin agar as a lipolysis test medium must now be questioned.

Tween has for long been commonly regarded as a useful substrate for demonstrating lipase. Archibald (1946) recommended the use of Tween 20 for the determination of lipase activity, because it was "soluble" and the enzyme and substrate would therefore be in the same phase whereas when emulsions of triglycerides etc. were used the substrate and the enzyme were in different phases. Sierra (1957) extended the use of Tween to the demonstration of bacterial enzyme systems. C.bovis grew well on Tween 20 agar and on Tween 80 agar, with the production of the zones of precipitation regarded by Sierra (1957) as indicative of lipolysis. This interpretation is, however, open to question. The current nomenclature (Recommendations, 1965) for enzyme reactions involving noncholine carboxylic esters, defines a lipase (Enzyme Commission number 3.1. 1.3) as an enzyme that acts on water-insoluble esters or fats at the ester/water interface in emulsions. It has been pointed out (Sarda and Desnuelle, 1958; Desnuelle, 1961; Desnuelle and Savary, 1963) that a substrate intended for the detection of lipase must therefore form an emulsion in water and not a solution. Water soluble substrates (e.g. p-nitrophenyl acetate) have been used in techniques intended to detect lipase activity but these are typically substrates for esterases and may not be acted upon by lipase (Desnuelle, 1961). Although the polyoxyethylene sorbitan fatty acid esters (Tween) form "solutions" in water that exhibit some of the properties of colloids e.g. exhibiting a Tyndall cone effect when illuminated with a suitable light source, these substrates can be acted upon by carboxyl-

esterases as well as by lipases (Seligman and Nachlas, 1950).

The interpretation of enzymic action on Tweens thus depends upon the exact physico-chemical nature of aqueous dispersions or solutions. A true lipase would only be activated by being adsorbed on to its substrate at the water/substrate interface. Does such an interface, that is one capable of causing activation of a lipase, exist in aqueous solutions of Tween?

A non-ionic surface-active agent usually consists of a water-insoluble lipophilic compound which is made water-soluble by the introduction of ethylene ether groups into the compound (Schwartz and Perry, 1949). The greater the number of ethylene ether groups the more hydrophilic the product becomes, until, with the inclusion of 12-15 ethylene ether groups per molecule, the compound becomes completely water-soluble. Tweens are fatty acid esters of anhydrosorbitols which have been solubilized by etherifying the free hydroxyl groups with ethylene oxide. When a non-ionic surface-active agent such as a Tween is dissolved in water, micelles are formed when the concentration exceeds the critical micelle concentration (CMC) for the specific surfactant. The CMC for most non-ionic surface-active agents lies between 0.0025 and 0.025% (Becher, 1959; Schwartz, Perry and Berch, 1958) and the CMC of Tween 20 was found by Becher (1959) to be 0.015%. If the CMC of Tween 80 were similar to that of Tween 20, both would be well below the concentration of Tweens used in Tween agar. The two main types of micelle formed by non-ionic surfactants are the spherical and lamellar, but there have also been reports of double layer micelles and rod-shaped micelles (Schwartz et al, 1958). It is obvious that

such micelles are greatly different from the globules of the internal phase of an emulsion (one difference being the presence of water within the micelle), and it is difficult to predict whether the physicochemical characteristics of a micellar system are such as to allow of activation of a lipase. Further research is required into the characteristics of the micelles of Tween and their effect upon pure preparations of lipases and esterases. One of the most useful techniques available for obtaining homogeneous enzyme preparations is zone electrophoresis and starch gel and polyacrylamide gel electrophoresis have been used in this investigation (see Section F) in an endeavour to elucidate these reactions with respect to C.bovis.

In the absence of the necessary detailed knowledge of the micellar structure of Tweens and their effect upon the hydrolytic enzymes, a number of conflicting premises have been made by various workers, and the situation has been further aggravated by the different definitions of lipases and esterases that have been employed.

For example, Gomori (1952) suggested that Tween 20, Tween 40 and Tween 60 were general substrates for lipase and for carboxylesterase whereas the unsaturated Tween 80 was a substrate specific to lipase, although the characteristics of lipase and esterase listed by Gomori (some of which are shown in Table 30 of Section F) did not lend support to his suggestions. On the other hand, Desnuelle and Savary (1963) considered the Tweens to be probably substrates for esterase rather than for lipase. The reaction produced by C.bovis on Tween 20 agar and Tween 80 agar therefore need notnecessarily be indicative of an

enzyme capable of hydrolysing triglycerides of long chain fatty acids.

Butter-fat agar supported good growth of C.bovis, with 63 of the 123 strains tested producing some degree of colour reaction when treated with saturated copper sulphate solution after incubation. The growth of the C.bovis isolates on butter-fat agar was characterized by pigment formation, the (nondiffusible) pigment being white, cream or yellow. This contrasted sharply with the growth of C.bovis on all other media used in this investigation, in which the colonies or growth of all the isolates were always greyish in colour. There appeared to be a correlation between the pigmentation and the copper sulphate reaction (Table 9), most of the yellow-pigmented strains giving no colour reaction with the copper sulphate. No zones of colour reaction developed in the medium surrounding the bacterial growth, but such zones were not particularly evident with lipolytic test cultures (Pseudomonas fluorescens, Alcaligenes viscolactis and Achromobacter lacticum).

Victoria blue margarine agar proved to be unsuitable for the growth of the isolates. With very heavy inocula it was possible to obtain slight reactions. The unsuitability of the medium was due to nutritional deficiency in the margarine (or an inhibitory effect of the margarine capable of being neutralized by the addition of serum,) rather than to an actual inhibitory effect of the Victoria blue, since moderately good growth of the isolates occurred on Victoria blue margarine serum agar and on Victoria blue butter-fat agar. The bacterial growths on both media without serum were coloured blue, suggesting that hydrolysis of

the fat had taken place. No zones of hydrolysis occurred on Victoria blue margarine agar. Only 10 strains produced zones on the Victoria blue butter-fat agar. These zones were so narrow that it is possible that they were illusory and due to the ill-defined boundary between the edge of the bacterial growth and the coarsely-surfaced opaque medium, since it was found impossible to remove the bacterial growth from the surface of the medium without removing the blue coloured medium.

The biochemical characteristics of the animal-parasitic corynebacteria according to Bergey's Manual (Breed et al, 1957) are shown in Table 10, and the biochemical characteristics of the animal-parasitic corynebacteria tested by Cowan and Steel (1965) are given in Table 11.

The characteristics of the isolates of C.bovis found in the present work to be comparatively constant (reactions obtained with 80% or more of the strains) were the ability to produce acid from glucose, fructose and glycerol; slight acid production from arabinose, xylose, rhamnose, mannitol and sorbitol; no acid production occurred with lactose, sucrose or salicin. Starch was not hydrolysed. Urea was hydrolysed. Growth occurred on serum agar, egg-yolk agar, Tween 20 agar, Tween 80 agar, and butter-fat agar, with evidence of hydrolysis of the substrate in the last three media. Catalase was produced. Gelatin was not liquefied. No growth usually occurred in litmus skim milk. Nitrite was not produced from nitrate. Hydrogen sulphide was not produced. Indole was not produced. No haemolysis occurred on blood agar.

These characteristics were unlike those of the organisms described by Breed and colleagues (1957)(Table 10) or by Cowan and Steel (1965) (Table 11), probably due to the use of unsuitable media by the workers on

TABLE 10: BIOCHEMICAL REACTIONS OF CORYNEBACTERIUM SPP. ACCORDING TO BREED ET AL (1957)

	Acid produced from:																	Starch hydrolysis		Indole production	H_2S production	NH_3 production	NO_3^- reduction	Urease	Gelatin liquefaction (nutrient gelatin or serum gelatin)	Pigment	Haemolysis	Catelase	Growth on ordinary (plc) agar	Litmus milk	Tributyrin agar
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Starch agar	Starch serum water											
C.diphtheriae var. gravis[1]			+	V	+				V	V		V								+	-			+	-	-	Grey, cream yellow or reddish.	-			-
C.enzymicum					+				+	+		+									+			+	-	Colourless or cream			AC	
C.hoegii					+				-	+											-			-	-	Pink			+	OH	
C.striatum[2]	-	-	+	V(+)	+	+	-	V(+)	V(+)	V	-	V	-	-	V	-	-	-		V(+)	-	+ or -	-	-	-	V	White or diffusible yellow-green	+	+	+	- (or late R)	
C.pseudotuberculosis[3]		V	+	+	+	+		+	+	+														-	-	Cream yellow or orange			+	-	
C.xerosis			+	+	+				+	+											-			-	-	Grey			+	-	
C.pyogenes[4]		+			+			+		+	-				-		-				-			+	+		+		-	ACP	
C.muriseptieum	-		+	+	+			+	+	+					+						+	+								A	
C.renale			V		+	V		-	-	-					-									-	Greyish or cream				CR, P &OH	

Notes: † C.diphtheriae var. mitis is haemolytic and does not produce acid from starch, D.diphtheriae var. intermedius is not haemolytic and does not produce acid from starch. C.ulcerens Gilbert and Stewart considered identical with C.diphtheriae Lehmann & Neumann.

2 Voges-Proskauer test, and methyl red, negative. Found in the human throat and also isolated from udders of cows with mastitis. Considered identical with C.flavidum Holland (J.Bact., 1920, 5, 218), according to description in 5th edition of Bergey (1939).

3 Considered synonymous with Bacillus tuberculosis ovis Lehmann & Neumann. Closely related serologically with C.renale (Merchant, 1930).

4 Carbohydrate reactions tested in serum broths.

(Continued).

TABLE 10:

BIOCHEMICAL REACTIONS OF

CORYNEBACTERIUM SPP.

ACCORDING TO BREED ET AL.(1957)

TABLE 10: (Continued)

	Acid produced from:																	Starch hydrolysis		Indole production	H_2S production	NH_3 production	NO_3^- reduction	Urease	Gelatin liquefaction (nutrient gelatin or serum gelatin)	Pigment	Haemolysis	Catalase	Growth on ordinary (plain) agar	Litmus milk	Tributyrin agar
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Starch agar	Starch serum water											
C.kutscheri	-		-	-	+		-	-	-	-	-	-	-	-	-	-	-	-			-	-	-	-	-	white	-			-	-
C.phocae[5]	-	±	+	±	+		V	±	+	+		±		-	±	-	+	±		+	-	-	-	-	-	white	-	-		-	-
C.pseudodiphtheriticum[6]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			-		+	+	-	grey or cream				-	-
C.equi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			-	+	-	+	-	tan, yellow or pink or red	- or ±			- or OH	
C.bovis[7]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			-			-		grey				OH	±
C.paurometabolum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			-	±		-	+	grey or white	±			OH	
C.nephridii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			-	-	+	-	-	grey	-		+	OH	
C.vesiculare	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	+	-	-	orange	-			OH	

Notes: 5 Carbohydrate reactions tested in serum broths; glucose, fructose, sucrose and salicin breakdown causes clotting of the medium in 24 hours at 37°C. Voges Proskauer and methyl red positive. Although catalase-negative, C.phocae is aerobic and facultative.

6 Synonymous with C.hofmannii

7 Evans considers Bacterium lipolyticus Evans to be identical with C.bovis

TABLE 10: (Continued)

TABLE 11: BIOCHEMICAL REACTIONS ACCORDING TO COWAN AND STEEL (1965)

	Acid from[1]:															Starch hydrolysis										
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol	Starch agar	Starch serum water	Indole production	H_2S production	NH_3 production	NO_3^- reduction	Urease	Gelatin liquefaction	Pink pigment	Haemolysis	Catalase
C.diphtheriae var. gravis					+			-	+	-							-			+				+	-	-	-	-	+
C.ulcerans					+			-	V	-							-			+				-	+	+	-	+	+
C.xerosis					+			-	+	+							-			-				+	-	-	-	-	+
C.renale					+			V	V	-							-			V				+	+	-	-	V	+
C.ovis					+			V	+	V							-			+				V	+	-	-	V	+
C.bovis					-			-	-	-							-			-				-	-	-	-	-	+
C.equi					-			-	-	-							-			-				+	+	-	+	-	+
C.hofmannii					-			-	-	-							-			-				+	+	-	-	-	+
C.pyogenes					+			+	+	V							-			+				-	-	+	-	+	-
C.haemolyticus					+			+	+	+							-			V				-	-	+	-	+	+
C.murium					+			-	+	+							V			+				+	V	-	-	-	+

[1] Cowan & Steel make the proviso that the basal medium for carbohydrate tests may be more important with corynebacteria than with other organisms.

TABLE 11: BIOCHEMICAL REACTIONS ACCORDING TO COMAN AND STEEL (1965)

whose results their descriptions were based. The reactions of C.bovis NCTC 3224 obtained in this work were very similar to those of the isolates, the outstanding differences being the inability of C.bovis NCTC 3224 to produce acid from glycerol or to hydrolyse urea, and its ability to produce slight acid from lactose.

Knight (1955) suggested that nutritional characteristics if sufficiently constant could be as important in delineating species as the morphological and biochemical characteristics more usually employed. Only the type cultures of C.hofmannii, C.bovis and C.pyogenes exhibited a requirement for serum, egg-yolk or Tween, in common with the C.bovis isolates. Corynebacterium hofmannii and C.pyogenes were considerably different from the C.bovis isolates and C.bovis NCTC 3224 in their biochemical characteristics.

The isolates obtained in this work therefore form a homogeneous group correctly designated as C.bovis Bergey et al, 1923, in spite of the apparent discrepancies in the biochemical reactions compared with those described by Breed and colleagues (1957). Although Jayne-Williams & Skerman (1966) isolated organisms corresponding to C.ulcerans from the bovine udder, none of the isolates in the present work corresponded with C.ulcerans (typically starch-hydrolysing, urea-hydrolysing, gelatin-liquefying, haemolytic) or with the atypical strains of C.ulcerans reported by Jebb and Martin (1965) one of which was unable to produce acid from starch and the other unable to hydrolyse urea.

APPENDIX: BIOCHEMICAL REACTIONS OF ISOLATES AND TYPE CULTURES (TABLE 12)

Key to symbols used:

1. Acid production from carbohydrates and starch:

   +, pH 5 or lower produced (this is followed by the number of days incubation required for pH to be reached).

   ±, pH of 5.5 - 7 produced.

   -, no acid produced, no change in pH.

   OH, alkaline reaction.

   ..., not tested.

2. ++, good growth; +, moderate growth; ±, slight growth;

   (±), growth only in the region of a heavy inoculum;

   -, no growth; ..., not tested.

3. ++, strong reaction or broad and distinct bands;

   +, moderate reaction or narrow bands;

   ±, slight reaction; (±), slight reaction or occurring only in the region of the heaviest inoculum; -, no reaction; ..., not tested.

4. Z, zone of colour reaction in the medium surrounding the bacterial growth;

   ++, deep colour reaction in the bacterial growth; +, moderate colour reaction in the bacterial growth;

   ±, slight colour reaction in the bacterial growth; -, no reaction;

   ..., not tested.

5. ++, pronounced blackening of lead acetate paper; +, moderate blackening of lead acetate paper; ±, slight blackening of lead acetate paper; -, no reaction.

   ..., not tested

6. * orange-red, brick red or brown colour with Nessler's reagent;

±, light orange or orange-yellow colour; -, no reaction; ..., not tested.

7. +, positive reaction; -, no reaction; ..., not tested.

8. A, acid reaction; C, clot or coagulation; R, reduction of litmus and loss of colour; G, gas produced; OH, alkaline reaction.

9. W, white pigment; C, cream pigment; Y, yellow pigment; T, tan pigment; L, lemon pigment.

TABLE 12.

APPENDIX TO SECTION D.

BIOCHEMICAL REACTIONS OF ISOLATES AND TYPE CULTURES.

	Acid production[1] from:																	
	Arabinose	Xylose	Fructose	Galactose	Glucose	Mannose	Rhamnose	Lactose	Maltose	Sucrose	Raffinose	Glycerol	Adonitol	Dulcitol	Mannitol	Sorbitol	Salicin	Inositol
Column number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
C.bovis isolates																		
2	±	-	+2	+4	±	-	±	-	±	+1	-	+5	-	-	±	±	-	OH
3	±	±	+2	+3	+4	±	±	-	-	-	OH	+5	OH	-	-	±	OH	-
4	-	±	+2	+4	±	±	±	-	-	-	-	+5	-	-	±	±	OH	OH
5	-	±	+3	+3	+3	±	±	+6	±	-	-	+5	-	-	-	±	OH	-
6	-	±	+2	±	+3	-	±	-	-	-	±	+4	-	OH	-	±	OH	OH
9	-	±	+2	±	+4	-	-	-	±	OH	-	+3	-	OH	±	±	OH	-
10	-	±	+2	-	+4	-	±	-	+18	-	-	+5	OH	-	±	±	OH	OH
11	-	±	+2	±	+4	±	±	-	-	-	-	+3	-	-	±	±	OH	OH
37	±	-	+3	+4	+5	±	±	-	+39	-	-	+5	-	-	±	±	OH	-
37M	±	±	...	...	+4	...	±	OH	...	-	-	...	-	-	±	±	...	-
38	±	±	+2	±	+5	-	+8	-	-	-	-	+3	-	-	±	±	OH	-
40	-	±	+2	-	±	-	±	-	-	-	±	+5	-	-	±	±	OH	-
41	±	±	+4	+2	+5	-	±	-	±	OH	-	+3	±	±	±	±	+2	±
44	-	±	+3	-	+4	-	±	-	+39	-	-	+4	-	OH	±	±	OH	-
45	±	±	+2	±	±	±	±	-	-	-	-	+5	-	OH	±	±	OH	-
51	±	±	+3	±	+3	-	±	-	-	-	-	+4	-	-	±	±	OH	-
52	-	±	+2	±	+4	-	±	-	-	-	-	+3	-	-	±	±	OH	OH
53	±	±	+3	-	+4	-	+14	-	OH	-	±	+3	±	-	-	±	OH	±
54	...	...	+3	±	+5	-	...	-	±	-	...	+7	...	...	...	...	OH	...
55	...	...	+4	-	+6	-	...	-	±	-	...	+18	...	...	...	...	OH	...

	Starch agar								Casein agar		Liquefaction of gelatin					Eggyolk agar		Egg-yolk broth	
	Growth[2]	Reaction[3]	Acid in starch serum water[1]	Production of indole[7]	Production of H_2S[5]	Production of NH_3[6]	Production of NO_3^-[7]	Hydrolysis of urea[7]	Growth[2]	Reaction[3]	Nutrient gelatin, 22°C[7]	Nutrient gelatin, 37°C[7]	Charcoal-gelatin discs[7]	Liquefaction of serum[7]	Litmus skim milk[8]	Growth[2]	Reaction[3]	Growth[2]	Reaction[3]
Column number:	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
C.bovis isolates																			
2	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	++	-	++	-
3	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-
4	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-
5	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-
6	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-
9	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	A	++	-	++	-
10	(±)	-	-	-	-	±	-	+	-		-	-	-	-	-	++	-	++	-
11	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	++	-	++	-
37	+	-	-	-	-	±	-	+	(±)	-	-	-	-	-	-	++	-	++	-
37M	...	...	-	-	-	±	-	...	...	...	-	-	-	-	...	++	-	++	-
38	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-
40	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-
41	-		-	-	-	±	+	+	(±)	-	-	-	-	-	-	++	-	++	-
44	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	++	-	++	-
45	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	++	-	++	-
51	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-
52	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-
53	-		-	-	-	±	-	...	+	-	-	-	-	-	-	++	-	++	-
54	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-
55	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-

	Tributyrin agar			Tween 20 agar		Tween 40 agar		Tween 60 agar		Tween 80 agar		Butter-fat agar			Victoria blue margarine agar		Vidal in butter fat agar		
	Growth[2]: Plain medium	Growth[2]: with serum	Reaction[3]	Growth[2]	Reaction[3]	Growth[2]	Reaction[3]	Growth[2]	Reaction[3]	Growth[2]	Reaction[3]	Growth[2]	Pigmentation[9]	Reaction ($CuSO_4$)[4]	Growth[2]	Reaction[4]	Growth[2]	Reaction[4]	Catalase[7]
Column number:	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
C.bovis isolates																			
2	...	...	...	...	...	...	...	...	...	...	...	++	W	++	(±)	-	(±)	±	+
3	(±)	(±)	(±)	++	++	-		+	-	++	++	++	Y	+	(±)	±	(±)	±	+
4	(±)	(±)	(±)	++	++	-		+	-	++	++	++	W	++	±	±	...	...	+
5	(±)	(±)	(±)	++	++	-		+	-	++	++	++	W	++	(±)	±	(±)	±	+
6	(±)	(±)	(±)	++	++	-		+	-	++	++	++	W	++	(±)	±	...	...	+
9	(±)	(±)	(±)	++	++	-		+	-	++	++	++	W	++	(±)	±	(±)	±	+
10	(±)	(±)	(±)	(±)	-	(±)	-	(±)	-	++	++	++	C	++	(±)	-	(±)	±	+
11	(±)	(±)	(±)	(±)	-	(±)	-	(±)	-	++	+	++	W	++	(±)	±	(±)	±	+
37	(±)	(±)	(±)	++	++	-		(±)	-	++	±	++	C	-	+	±	+	±	+
37M	...	...	...	...	...	...	...	...	...	...	...	++	C	-	+	±	+	±	+
38	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	C	-	+	±	+	±	+
40	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	C	+	...	...	...	...	+
41	(±)	(±)	(±)	++	++	-		(±)	-	++	++	++	C	++	(±)	±	+	±	+
44	(±)	(±)	(±)	++	++	-		(±)	-	++	++	++	C	++	(±)	±	...	...	+
45	(±)	(±)	(±)	++	++	(±)	-	(±)	-	++	++	++	W	++	-		-		+
51	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	+	±	...	...	+
52	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	(±)	±	(±)	(±)	+
53	(±)	(±)	(±)	++	++	-		±	-	++	++	++	C	++	(±)	±	(±)	±	+
54	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	-	(±)	±	(±)	±	+
55	(±)	(±)	(±)	++	++	-		±	-	++	++	++	C	-	+	±	...	...	+

TABLE 12: (Continued)

Column number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
C.bovis isolates																																																										
56	...	...	+2	+4	+5	±	...	-	±	-	...	+6	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(+)	(+)	(+)	++	++	-		±	-	++	++	++	C	++	(±)	±	(±)	±	+
57	...	...	+3	+4	+4	-	...	-	±	-	...	+11	...	...	...	...	OH	...	...	...	-	-	-	±	-	-	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	Y	-	(±)	±	(±)	±	+
58	...	...	+2	-	+6	-	...	-	+7	-	...	+5	...	...	...	...	OH	...	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	C	++	...	...	...	...	+
59	±	±	+2	±	+5	OH	±	-	OH	-	±	+4	±	±	±	±	OH	±	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	W	++	(±)	±	(±)	±	+
60	...	...	+2	+10	+5	-	...	-	±	-	...	+3	...	...	...	...	OH	...	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	W	++	(±)	±	+	+Z	
61	...	...	+2	+4	+5	±	...	OH	±	-	...	+7	...	...	...	...	OH	...	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	C	++	(±)	±	+	±	+
62	-	±	+2	+4	+4	±	±	-	±	-	±	+4	±	±	±	±	OH	±	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	W	++	(±)	±	+	±	+
63	±	±	+3	+3	+6	-	±	±	±	OH	±	+6	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	A	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	W	++	±	±	+	±	+
64	±	-	+6	-	+5	-	±	-	-	-	-	-	-	±	±	±	OH	±	-		-	-	-	±	-	-	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	Y	-	+	±	...	...	+
65	±	±	+3	+4	+5	-	±	-	-	...	±	+4	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
66	±	±	+2	+20	+3	-	±	-	-	-	±	+4	±	OH	±	OH	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	W	++	(±)	±	+	+Z	+
67	+3	±	+3	±	+4	±	±	-	-	-	±	+5	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	Y	-	+	±	+	±	+
68	±	±	+2	±	+3	±	±	-	-	-	±	+4	-	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	C	+	+	±	+	±	+
69	±	±	+3	±	+4	±	±	-	±	-	±	+6	-	±	±	±	±	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	+	±	+	+	+
70	-	±	+3	+39	+4	-	±	-	±	-	OH	+5	±	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	+	+	±	+	+	+
71	±	±	+3	+3	+3	±	±	-	±	-	±	+11	OH	±	+35	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	+	+	±	+	+	+
72	±	±	+3	±	+3	±	±	-	±	OH	±	+5	±	±	±	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	-	+	±	+	+	+
73	±	±	+4	+18	+4	±	±	-	±	-	±	+5	±	±	±	±	OH	±	...	...	-	-	-	±	-	+	...	...	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	W	++	...	...	...	...	+
74	±	±	+3	-	+4	±	±	-	-	-	±	+5	±	±	±	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	ACR	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	C	++	...	...	...	...	+
75	±	±	+5	+22	+5	...	±	-	±	-	±	+5	±	±	±	-	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	-	(±)	±	-		+
76	±	±	+2	±	+4	-	-	±	-	-	-	+3	-	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	(±)	±	±	+Z	+
77	±	±	+3	-	+4	-	±	-	±	OH	-	+14	±	±	±	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	...	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	+	...	...	...	...	+
78	±	±	+3	-	+4	OH	±	-	±	-	-	+5	±	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	+	(±)	±	(±)	±	+
79	±	±	+4	+6	+3	±	±	-	-	OH	OH	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	-	(±)	±	(±)	±	+
80	±	±	+4	±	+3	±	±	±	-	OH	±	+7	±	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	+	(±)	±	+	±	+
81	±	±	+2	±	+4	±	±	-	±	OH	±	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	Y	-	(±)	±	+	+Z	+
82	±	±	+4	±	+3	-	±	±	±	-	±	+5	-	-	±	±	OH	OH	-		-	-	-	±	-	+	+	-	-	-	-	-	A	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	±	++	Y	+	...	...	...	...	+
83	±	±	+3	±	+3	±	±	-	±	-	±	+6	±	±	±	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	+	++	C	++	...	...	...	...	+
84	±	±	+3	-	+4	-	±	-	-	-	±	+4	±	±	±	±	OH	±	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	...	...	...	...	+
85	...	...	+4	-	+4	-	...	-	±	-	...	+5	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	...	...	...	...	+
86	...	...	+3	-	+4	-	...	OH	±	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	+	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	...	...	...	...	+
87	...	...	+2	±	+3	-	...	-	...	-	...	+3	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-		±	-	++	++	++	W	++	(±)	±	+	+Z	+

TABLE 12: (Continued)

Column numbers:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
Cabergia isolates																																																									
88	...	...	+2	±	+4	...	...	±	±	OH	...	+3	...	...	...	...	OH	...	-	-	-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	-	-	±	-	++	+	++	Y	+	(±)	±	+	+±	+
89	...	...	+2	OH	+6	OH	...	-	-	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	+	++	Y	-	(±)	±	+	±	+
90	...	...	+2	-	+4	±	...	-	-	-	...	+7	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	+	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
91	...	...	+2	±	+6	OH	...	-	+7	-	...	+5	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	(±)	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
92	...	...	+2	-	+4	±	...	-	-	-	...	+3	...	...	...	...	OH	...	-		-	-	-	±	-	+	...	...	-	-	-	-	...	++	-	++	-	(±)	(±)	(±)	...	...	...	...	...	...	...	...	++	Y	-	(±)	±	-		+
93	...	...	+2	+4	+4	±	...	OH	-	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
94	...	...	+2	±	+4	±	...	OH	±	-	...	+3	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	+	±	+	±	+
95	...	...	+2	+4	+5	OH	...	-	-	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	+	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	(±)	-	++	++	++	Y	-	(±)	±	+	±	+
96	...	...	+3	±	+5	±	...	-	OH	-	...	+3	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	-	-	(±)	-	++	++	++	Y	-	(±)	±	±	±	+
98	±	±	+2	-	+3	±	±	-	OH	-	-	+4	±	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	-	-	(±)	-	++	++	++	Y	-	+	±	+	±	+
100	±	±	+2	±	+4	OH	±	-	±	-	OH	+3	±	-	±	±	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	+	±	(±)	±	+
101	...	...	+2	±	+4	OH	...	OH	±	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	(±)	±	...	...	+
102	...	...	+4	...	+4	OH	...	-	±	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	(±)	±	...	...	+
104	...	...	+2	±	+3	±	...	OH	OH	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	+	C	-	(±)	±	(±)	±	+
105	±	±	+2	+4	+3	+	±	-	±	-	±	-	±	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	C	-	(±)	±	(±)	±	+
106	±	±	+2	OH	+4	±	-	-	...	-	-	+4	-	-	±	-	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	C	-	...	...	...	...	+
107	±	±	+2	±	+3	±	±	-	-	-	-	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	(±)	-	++	++	++	C	-	(±)	±	(±)	±	+
108	±	±	+2	+4	+3	±	±	OH	OH	OH	OH	+3	±	OH	±	±	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	(±)	-	++	++	+	C	-	(±)	±	(±)	±	+
109	±	±	+2	±	+4	±	±	OH	-	±	-	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	+	C	-	(±)	±	(±)	+±	+
110	±	±	+2	OH	+4	±	±	-	OH	OH	-	+4	-	-	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	±	-	++	++	++	Y	-	(±)	±	(±)	±	+
111	±	±	+3	±	+3	±	±	±	±	-	-	+2	-	-	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	+	++	C	+	+	+	...	...	+
112	±	±	+2	-	+4	±	±	-	-	-	-	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	...	...	++	++	++	C	-	...	...	...	...	+
113	±	±	+2	±	+3	±	±	-	...	-	-	±	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	...	...	++	++	++	Y	-	+	±	+	±	+
114	...	...	+4	...	+4	±	...	±	±	-	...	+5	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	+	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	...	...	++	±	++	C	+	+	±	+	+	+
115	±	±	±	-	+3	-	±	±	...	-	-	+7	±	-	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	...	...	++	++	++	W	++	+	±	+	±	+
116	...	...	+2	-	+4	±	...	±	OH	-	...	+7	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	...	...	++	++	++	C	++	(±)	±	...	...	+
117	±	±	+3	+7	+6	OH	±	-	-	-	-	+3	-	-	-	±	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	...	...	++	++	++	C	-	(±)	±	...	...	+
118	±	±	+2	-	+5	±	±	-	OH	-	-	+3	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
119	±	±	+2	-	+5	±	±	±	OH	-	-	+2	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	-	++	W	-	(±)	±	+	±	+
120	...	...	+2	±	+5	±	...	...	+7	-	...	...	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	++	-	++	-	(±)	(±)	(±)	++	++	-	±	-	++	++	++	Y	-	(±)	±	+	±	+
121	±	±	+2	+7	+5	±	±	+6	-	-	-	+3	-	-	-	±	OH	-	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	++	-	++	-	...	...	...	...	...	...	...	...	...	...	++	W	++	(±)	±	-		+

TABLE 12: (Continued)

Column number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
C.bovis isolates																																																									
122	±	±	+2	±	±	±	±	-	...	-	-	+3	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	...	...	...	(±)	±	...	...	+
123	±	±	+2	±	+4	±	±	+6	...	-	±	+3	-	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	+	C	+	(±)	±	-		+
124	±	±	+3	+10	+6	OH	±	-	+50	-	±	+3	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	W	+	(±)	±	+	+	+
125	...	...	+2	+4	+3	±	...	+6	±	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	C	+	(±)	±	...	...	+
127	...	...	+3	+10	+7	OH	...	-	±	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	W	-	(±)	±	±	+Z	+
129	±	±	+3	+4	+3	OH	±	±	±	-	±	+4	-	-	-	±	OH	±	-		-	-	-	±	±	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	Y	+	(±)	±	+	±	+
130	±	-	+2	+4	+4	±	-	±	±	-	-	+4	-	±	-	±	OH	±	-		-	-	-	±	±	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	C	↔	±	±	...	...	+
131	...	...	+3	+7	+6	+33	...	±	±	-	...	+5	...	...	...	...	OH	...	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	...	...	...	±	±	...	...	+
132	...	...	+3	+33	+7	±	...	-	±	-	...	+5	...	...	...	...	OH	...	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	...	...	...	(±)	±	...	...	+
133	±	-	+3	-	+6	-	±	-	+12	+1	-	+5	-	±	±	±	OH	±	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	C	↔	(±)	±	+	+	+
134	...	...	+2	-	±	+12	...	-	-	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	Y	-	(±)	±	+	+Z	+
135	...	...	+3	+33	±	-	...	-	-	-	...	+4	...	...	...	...	OH	...	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	W	↔	(±)	±	+	+Z	+
136	±	±	+3	+7	+6	±	±	-	-	-	-	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	C	↔	(±)	±	...	...	+
137	±	±	+2	+5	+4	...	±	-	±	-	±	+4	-	±	-	±	OH	±	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	-	↔	↔	↔	Y	-	(±)	±	+	±	+
138	±	±	+3	+4	+5	±	±	-	±	-	-	+4	-	-	-	-	OH	-	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	Y	-	(±)	±	...	...	+
139	±	±	+3	+7	+6	±	±	±	±	-	±	+6	±	-	±	±	OH	-	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	+	Y	-	(±)	±	+	±	+
140	±	±	+3	+5	+6	-	±	-	±	-	±	+4	-	-	±	±	OH	±	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	W	+	(±)	±	+	±	+
141	±	±	+3	-	+9	-	±	-	-	-	±	+5	±	±	±	±	OH	±	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	C	-	(±)	±	+	±	+
142	±	±	+3	±	+7	±	±	-	+14	-	±	+6	-	±	±	±	OH	OH	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	(±)	-	↔	↔	↔	W	-	(±)	±	-		+
143	±	±	+3	±	+6	±	±	-	±	-	-	+6	-	-	OH	±	OH	-	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	-		↔	↔	↔	C	-	±	±	...	...	+
144	±	±	+3	+33	+11	±	±	-	±	-	-	+4	±	-	±	±	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	-		↔	±	↔	Y	-	±	±	...	...	+
145	±	±	+3	+4	+4	-	±	-	±	-	±	+4	OH	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	±	±	↔	±	↔	W	↔	(±)	±	+	+Z	+
146	±	±	+3	-	+6	±	-	-	±	-	-	+4	-	-	+56	±	OH	OH	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	-		↔	-	↔	Y	-	(±)	±	+	+Z	+
147	±	±	+2	±	+4	±	±	-	±	-	-	+4	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	-		↔	+	↔	Y	-	(±)	±	+	±	+
148	±	±	+3	±	+5	±	±	-	-	-	±	+4	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	(±)	±	±	±	↔	↔	W	↔	(±)	±	(±)	+Z	+
149	±	-	+3	+4	+5	±	±	-	±	-	-	+5	-	±	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-	-		↔	±	↔	Y	-	(±)	±	+	+Z	+
150	±	±	+3	-	+3	±	+9	±	-	-	-	+6	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	...	(±)	↔	-	-	±	-	↔	↔	↔	Y	+	(±)	±	+	±	+
151	±	±	...	-	±	-	±	-	±	-	±	+7	OH	-	+35	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	...	(±)	↔	±	-	±	-	↔	-	↔	Y	-	(±)	±	+	±	+
152	±	±	+3	±	+5	±	±	-	±	-	±	±	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	...	(±)	↔	-	(±)	-	±	-	↔	-	W	+	(±)	±	...	...	+
153	±	±	+4	+6	+7	-	+	-	+14	-	±	+6	±	±	±	±	OH	±	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	...	(±)	↔	+	-	±	-	↔	↔	↔	Y	-	(±)	±	...	...	1
155	±	±	+3	+33	±	-	±	-	±	-	±	+5	±	-	±	±	OH	±	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	▲	↔	-	↔	-	(±)	(±)	-	↔	↔	-	(±)	-	↔	↔	↔	Y	-	(±)	±	(±)	±	+
156	±	±	+3	+33	-	-	±	±	±	-	±	+4	-	-	±	±	OH	±	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	-	↔	↔	-	(±)	-	↔	↔	↔	Y	-	(±)	±	(±)	±	+

TABLE 12: - (Continued)

Column number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56
157	±	±	+2	+8	+7	±	-	±	±	-	±	+5	-	-	±	±	OH	±	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	+	-		(±)	-	↔	↔	↔	Y	-	(±)	±	+	±	+
158	-	±	+3	+14	+5	±	±	-	±	-	-	+7	-	-	-	±	OH	±	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	-	↔	-	-		(±)	-	↔	↔	↔	C	-	(±)	±	...	...	+
159	±	±	+3	+12	±	-	±	-	±	-	-	+4	±	-	±	±	-	-	(±)	-	-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	-	↔	↔	-		(±)	-	↔	↔	↔	W	↔	(±)	±	+	±	+
160	±	±	+3	+12	+6	±	-	-	±	-	-	+6	-	-	±	±	OH	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	-	↔	↔	-		(±)	-	↔	↔	↔	W	↔	(±)	±	...	...	+
161	±	±	+3	+5	+4	±	±	-	+6	-	OH	+5	-	-	-	-	-	-	-		-	-	-	±	-	+	±	-	-	-	-	-	-	↔	-	↔	-	(±)	(±)	(±)	↔	↔	-		(±)	-	↔	↔	↔	Y	-	(±)	±	...	...	+
162	±	±	+3	+4	+4	±	±	-	±	-	-	+4	±	±	±	±	OH	±	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	...	...	...	...	...	...	...	...	...	...	...	...	↔	C	+	(±)	±	(±)	±	+
163	±	±	+4	+7	+4	±	±	+33	+12	-	-	+4	-	-	-	±	OH	-	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	...	...	...	...	...	...	...	...	...	...	...	...	+	C	-	...	...	...	...	±
165	±	±	+3	±	+5	-	±	-	±	-	-	+4	-	-	±	±	OH	-	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	....	...	...	...	...	...	...	...	...	...	...	...	↔	Y	-	(±)	±	(±)	±	+
166	-	-	+3	±	+6	+7	-	-	+14	-	-	+4	OH	OH	-	±	OH	OH	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	...	...	...	...	...	...	...	...	...	...	...	...	↔	Y	-	-		-		+
167	±	±	+3	±	+7	-	±	-	-	-	±	+4	-	-	±	±	OH	-	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	...	...	...	...	...	...	...	...	...	...	...	...	↔	W	↔	(±)	±	(±)	±	+
169	-	-	+3	+5	±	OH	-	-	+7	-	-	+4	-	-	-	±	OH	-	...	...	-	-	-	±	-	+	...	...	-	-	-	-	...	↔	-	↔	-	...	...	...	...	...	...	...	...	...	...	...	...	↔	C	-	...	...	...	...	+
Equine diphtheroids:																																																								
170	...	...	+1	+1	+1	+2	...	+1	+1	+1	...	OH	...	...	...	...	+1	...	...	...	...	...	-	...	-	...	...	-	...	...	...	-	...	±	-	±	-	...	...	...	...	...	...	...	...	...	...	...	...	...	±	-	-	...	...	...	...	...
171	...	...	+2	+2	+2	+2	...	+2	+2	+3	...	+3	...	...	...	...	OH	...	...	...	...	...	-	...	-	...	...	-	...	...	...	-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
172	...	...	+2	+2	+2	+2	...	+2	+2	+3	...	+7	...	...	...	...	OH	...	...	...	...	...	-	...	-	...	...	-	...	...	...	-	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Human diphtheroid:																																																									
173	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	-	...	±	-	±	-	...	...	...	...	...	...	...	...	...	...	...	...	±	-	-	...	...	...	...	...
Nocardial isolates:																																																										
7	-	±	±	+2	+1	-	+3	OH	±	+2	-	OH	-	-	-	-	OH	OH	↔	±	-	-	-	±	-	-	↔	-	-	-	-	-	-	↔	-	↔	-	↔	↔	+	↔	↔	↔	↔	↔	-	↔	-	+	T	-	±	-	+	-	+
17	±	±	+4	+2	+3	-	+8	+3	+4	+3	-	OH	-	-	-	-	OH	-	↔	+	-	-	-	±	+	-	↔	-	-	-	-	-	-	↔	-	↔	-	↔	+	+	↔	↔	↔	↔	↔	-	↔	-	+	T	-	+	±	+	±	+
39	...	...	±	-	±	±	...	-	±	-	...	-	...	...	...	...	OH	...	↔	↔	-	-	-	±	+	-	↔	-	+	-	-	-	OH	↔	-	↔	-	±	±	(±)	±	-	↔	↔	↔	↔	(±)	-	↔	S	-	+	±	+	-	+
46	±	±	+2	+2	+1	+4	+9	+1	+3	+1	±	-	OH	-	-	±	OH	±	↔	-	-	-	-	±	+	-	+	-	-	-	-	-	-	↔	-	↔	-	↔	±	↔	↔	↔	↔	↔	↔	-	↔	-	±	T	-	+	±	+	±	+
47	±	±	+2	+1	+2	+2	+4	+1	+3	+1	±	OH	±	±	±	±	OH	-	↔	-	-	-	-	±	+	-	↔	+	-	-	-	-	-	↔	-	↔	-	↔	±	↔	↔	↔	↔	↔	↔	-	↔	-	±	T	-	+	±	+	±	+
48	±	±	+2	±	+4	±	+19	-	-	-	±	+4	±	±	±	±	OH	±	-		-	-	-	±	-	+	(±)	-	-	-	-	-	-	↔	-	↔	-	±	±	-	↔	↔	-		(±)	-	↔	↔	±	T	-	+	±	+	±	+
49	±	±	+3	+2	+1	-	+3	+1	±	+1	-	OH	-	-	-	-	OH	±	↔	-	-	-	-	±	-	+	↔	-	-	-	-	-	-	↔	-	↔	-	↔	±	↔	↔	↔	↔	↔	↔	-	↔	-	±	T	-	+	±	+	±	+
50	±	±	+2	+2	+1	-	+2	+1	+4	+1	-	OH	-	OH	-	-	OH	-	↔	-	-	-	-	±	±	-	↔	±	-	-	-	-	-	↔	-	↔	-	↔	±	↔	↔	↔	↔	↔	↔	-	↔	-	±	T	-	+	±	+	±	+
Microbacterium isolates:																																																									
12	±	±	-	±	±	±	±	-	±	-	±	-	-	-	OH	-	OH	-	+	↔	-	-	-	±	-	+	+	-	+	-	-	-	R	↔	-	↔	-	+	↔	+	↔	↔	↔	↔	↔	↔	↔	↔	L	-	+	-	+	-	+
13	±	±	-	±	±	OH	+35	-	-	-	OH	-	-	-	-	±	OH	-	+	↔	-	-	±	±	-	+	+	-	+	-	-	-	ACR	↔	-	↔	-	+	↔	+	↔	↔	↔	↔	↔	↔	↔	↔	L	-	+	-	+	-	+
14	-	±	-	-	±	OH	-	-	-	OH	-	-	-	OH	-	±	OH	-	+	↔	-	-	-	±	-	+	+	-	+	-	-	-	OH	↔	-	↔	-	+	↔	+	↔	↔	↔	↔	↔	↔	↔	↔	L	-	+	-	+	-	+
15	±	-	-	±	-	OH	±	-	-	-	±	OH	-	±	±	±	OH	±	+	↔	-	-	±	±	-	+	+	-	+	-	-	-	OH	↔	-	↔	-	+	↔	+	↔	↔	↔	↔	↔	↔	↔	↔	L	-	+	-	+	-	+
33	...	...	-	±	-	-	...	-	...	-	...	-	...	...	...	...	OH	...	↔	↔	-	-	±	±	-	+	↔	-	+	-	-	-	OH	↔	-	↔	-	+	↔	+	↔	↔	↔	↔	↔	↔	↔	↔	L	-	+	-	+	-	+
G.hoffmanii NCIB 231	-	-	OH	OH	OH	OH	-	OH	OH	OH	-	OH	-	-	OH	OH	OH	OH	↔	-	-	-	-	±	+	+	↔	-	-	-	-	-	-	↔	-	↔	-	±	±	-	↔	↔	±	+	↔	±	↔	-	↔	Y	↔	(±)	±	-		+

TABLE 12: (Continued)

Column number:	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	5
C.flavidum NCTC 764	±	-	+2	+3	+2	+2	+2	OH	-	+2	-	-	-	OH	-	±	OH	-	...	...	±	-	+	±	+	-	...	⋯	-	-	-	-	...	++	-	++	-	++	++	++	++	++	++	++	+	++	++	++	C	-	...	...	...	...	+
C.bovis NCTC 3224	±	±	+3	±	+4	±	±	±	+33	OH	±	-	±	±	±	±	OH	±	(±)	-	-	-	-	±	-	-	±	-	-	-	-	-	slight R	++	-	++	-	(±)	(±)	(±)	++	++	(±)	-	(±)	-	++	++	++	C	++	(±)	(±)	+	+	+
C.ovis NCTC 3450	±	±	+1	+5	+1	+2	-	-	+2	-	OH	±	OH	OH	OH	OH	OH	OH	++	+	OH	-	-	±	-	+	++	+	-	-	-	-	-	++	-	...	...	+	+	-	++	++	+	+	±	++	++	±	...	...	...	-		...	...	+
C.diphtheriae NCTC 3985	±	-	+1	+1	+1	+1	+8	OH	+1	OH	-	+3	-	-	OH	±	-	±	++	++	+8	-	+	+	+	-	...	⋯	-	-	-	-	...	++	-	++	-	+	++	+	++	++	++	+	++	-	++	±	++	C	±	...	...	...	...	+
C.pyogenes NCTC 6448	-	-	+3	+3	+3	±	-	+3	+2	+3	-	±	-	-	-	-	±	-	-		+49	-	-	++	-	-	±	++	+	-	-	+	AC	±	-	...	...	±	±	-	±	-	(±)	-	(±)	-	-		...	...	...	-		...	...	-
C.xerosis NCTC 7243	-	-	+3	+2	+2	+3	+4	OH	+2	+2	-	+4	-	OH	OH	-	OH	OH	++	+	-	-	-	-	-	-	...	⋯	-	-	-	-	...	++	-	++	-	++	++	++	++	++	(±)	-	(±)	-	++	++	++	W	++	(±)	±	++	-	+
NCTC 9755	-	±	±	±2	+4	-	-	-	-	±	-	±	-	-	OH	OH	OH	-	++	±	-	-	++	±	+	-	...	⋯	-	-	-	-	...	++	-	++	-	++	++	++	++	++	++	++	++	++	++	++	++	O	-	+	-	++	±	+
C.renale NCTC 7448	-	-	+2	-	+3	+18	+9	OH	±	OH	-	OH	-	-	OH	OH	OH	-	++	-	-	-	-	±	-	+	++	++	-	-	-	-	OH	++	-	++	-	++	+	++	++	++	+	++	++	-	++	±	++	W	-	(±)	±	+	-	+
C.ulcerans NCTC 7910	-	±	+2	±	+15	+3	+2	-	+3	±	OH	+9	OH	±	OH	±	OH	-	+++	+++	+2	-	+++	++	▬	+	...	⋯	+	-	-		...	++	-	++	++	++	++	±	++	+	++	+	++	++	++	+	++	C	-	...	...	(±)	-	+
Microbacterium lacticum NCIB 8540 tested at 30°	...	...	+5	+12	+6	+7	...	+4	+7	-	...	±	...	...	...	...	+18	...	+++	++	-	-	-	±	+	-	++	++	-	-	-	-	AC	++	-	++	-	±	±	-	++	-	++	-	...	...	++	-	±	-	-	++	-	-	+
tested at 37°	...	...	±	OH	-	OH	...	-	±	OH	...	OH	...	...	...	...	-	...	+++	++	-	-	-	±	-	-	++	±	-	-	-	-	-	++	-	++	-	±	±	-	-	±	-	±	-	(±)	-	±	-	-	-	-	-	+
NCIB 8541 tested at 30°	...	...	+2	+4	+2	+2	...	OH	+5	±	...	+6	...	...	...	...	+12	...	+++	-	-	-	-	±	-	-	++	+	-	-	-	-	-	++	-	++	-	+	±	-	++	-	++	-	...	...	++	-	±	-	-	++	-	-	+
tested at 37°	...	...	+4	+5	+4	+3	...	-	+4	+12	...	-	...	...	...	...	-	...	++	±	-	-	-	±	+	-	++	±	-	-	-	-	-	++	-	++	-	+	±	-	-	±	-	±	±	-	±	-	-	-	-	-	+
Corynebacterium isolates not C.bovis 103	...	...	+2	OH	±	+4	...	-	+18	+6	...	OH	...	...	...	...	OH	...	-		-	-	-	±	-	-	±	-	-	-	-	-	-	++	-	++	-	±	±	(±)	++	++	++	++	+	++	++	+	...	...	...	+	-	...	...	+
154	±	±	±	OH	+11	±	+35	-	OH	-	±	±	±	±	±	±	-	±	+++	-	-	-	+	++	+	+	++	-	-	-	-	-	R	++	-	++	-	++	±	++	++	++	++	++	++	++	++	-	+	W	-	+	±	+	-	+

NUTRITIONAL STUDIES

Introduction

As discussed in Section C the investigation into the cultural characteristics of *Corynebacterium bovis* indicated that serum, egg-yolk or butter-fat must be added to a basal nutrient medium such as tryptose lemco agar, for growth to occur. The inability of the organism to grow either in tryptose lemco agar to which activated charcoal had been added or in a nutrient broth (Panmede broth) to which albumin had been added suggested that the addition of serum etc. acted by fulfilling a nutritional requirement rather than by detoxifying any fatty acids that may be present in the basal medium. The growth of *C.bovis* on Tween 20 agar and Tween 80 agar was a further indication that this requirement may be for a fatty acid.

This requirement presumably could not be fulfilled by the casual presence of minute traces of fatty acids in nutrient media derived from cotton wool (Pollock, 1949; Hart *et al.*, 1962) or from meat extract or peptone (Pollock *et al.*, 1949), since *C.bovis* could not grow in Panmede broth to which albumin had been added, although the Panmede liver digest provided a balanced source of all the B-group vitamins and amino acids. Albumin does not protect bacteria from the toxic effects of fatty acids by irreversibly binding the fatty acid, but rather by keeping the free fatty acid concentration below the bacteriostatic level, the bound acid constituting a reserve that replaces the fatty acid utilized by the bacteria (Davis and Dubos, 1947; Johnson and Gary, 1963). The absence of growth by *C.bovis* on Tween 40 agar is further evidence that if

C.bovis possesses a requirement for unsaturated fatty acids, the fatty acids incidentally occurring in nutrient media are not able to satisfy this requirement since Tween 40 has also been found capable of detoxifying unsaturated fatty acids (Williams et al., 1947). The fatty acid requirements of corynebacteria may very from those of the oleic acid-requiring diphtheroids isolated by Pollock and his colleagues (1949) which obtained sufficient free fatty acid from the components of nutrient agar to be able to grow on this medium without supplementation, to those of the corynebacteria described by Silliker and co-workers (1963) which required high concentrations of unsaturated fatty acids and were able to grow profusely in the presence of 0.5 - 1% oleate without the need for detoxification. The corynebacteria described by Pollock and co-workers could not grow on nutrient agar to which charcoal had been added, presumably because the free fatty acids were adsorbed on to the charcoal.

In view of the difficulty in determining the growth requirements of C.bovis when empirical media are used, it was felt that the development of a chemically defined medium for the growth of Corynebacterium bovis was necessary.

The two common bases for considering the growth of micro-organisms in chemically defined media (or indeed in any media) are detectable (that is minimal) growth and normal growth. The determination of "normal growth" requires a standard of comparison, as well as a reproducible method of measuring growth. In the present investigation the standard of comparison representing normal growth was taken as the amount of

growth of a strain of C.bovis in a nutrient broth containing bovine serum, and a convenient method of growth measurement was by determining the turbidity of broth cultures using a nephelometer. The aim, therefore, was to obtain in chemically-defined media growth at least as good as that obtained in the serum-containing empirical medium, with particular reference to the effect of the presence or absence of fatty acids or compounds containing fatty acids. Since the supplements to empirical media which allowed the growth of C.bovis were also surface-active agents, the effects of other surface-active agents were studied in addition.

As pointed out by Mueller (1940), there are two ways in which the development of a chemically defined medium can be attempted. In the "synthetic" approach, various known and possible sources of nitrogen and carbon etc., and growth factors are used in different combinations until growth of the organism is obtained. The second approach (and the one favoured by Mueller in determining the nutritional requirements of C.diphtheriae) is "analytical", using the empirical medium in which the organism will grow as the starting point. This empirical medium should be broken up into a number of components, whose effect upon growth is then determined. Chemical extractions can then enable identification of the compounds involved. With the majority of organisms which can exist and grow in a variety of habitats the second, analytical approach would appear to offer the best chances of success. In the case of C.bovis its apparently restricted habitat suggested that the first method could be used for

the development of a defined medium, since reasonably complete analyses of bovine milk have been carried out. In addition, the adequacy of a serum-containing medium for the growth of C.bovis indicated that the development of a medium containing a substitute for serum would offer at least the minimal requirements for growth. Typical compositions of milk and of blood serum are shown in Table 13.

The first type of medium used in this investigation was a partially defined basal medium in which amino acids were provided largely by the inclusion of Casamino acids. On the basis of the results obtained using this medium, growth of the organisms was subsequently examined using fully defined media in which named amino acids and known vitamins and growth factors were added. Since the main object of this part of the investigation was to determine whether C.bovis had a requirement for fatty acids or fatty acid-containing compounds, the minimal requirements of C.bovis for amino acids and vitamins were not determined although a number of fully defined basal media incorporating various amino acids etc. were used.

A comparison of the growth of C.bovis in tryptose serum broth (TSB) dispensed (a) in 7 ml amounts in 6 x $\frac{5}{8}$ inch test tubes and (b) in 25 ml amounts in 100 ml Erlenmeyer flasks showed that oxygen supply was a severely limiting factor in the case of media dispensed in test tubes. Consequently the nutritional requirements of the selected strains were studied in liquid media dispensed in 25 ml or 50 ml amounts in 100 ml Erlenmeyer flasks incubated in shaking water baths (A. Gallenkamp and Co. Ltd., London), a procedure which

TABLE 13. COMPOSITION OF MILK AND SERUM. (a) AMINO ACIDS

Values in mg per 100 ml unless otherwise stated

Component	Bovine milk[1]	Bovine milk[2]	Bovine serum[3]	Human plasma[3]	Human plasma or serum[2]
Amino acid					
Alanine				3.4	2.57-5.37
α-Aminobutyric acid				0.3	
Arginine	125	120-160		1.62	1.10-3.58
Aspartic acid				0.03	
Cystine and cysteine				1.47	0.89-2.05
Glutamic acid				0.7	0.63-6.19
Glycine				1.5	1.25-2.29
Histidine	80	110-113		1.38	1.06-1.78
Isoleucine	213	210-290		1.34	0.98-2.22
Leucine	360	320-390		1.86	1.23-2.59
Lysine	260	230-310		2.19	2.11-3.79
Methionine	86	60-90		0.52	0.46-1.48
Ornithine				0.72	
Phenylalanine	173	150-220		0.99	0.74-2.02
Proline				2.36	
Serine				1.12	
Taurine				0.55	
Threonine	153	130-220		1.67	1.12-2.92
Tryptophan	50	40-80		1.27	0.66-1.50
Tyrosine				1.04	0.74-2.22
Valine	230	240-280		2.72	2.15-3.51
Asparagine				0.58	
Citrulline				0.49	0.38-0.59
Creatine		as N 2.45-5.62		0.39	0.32-0.72
Creatinine		as N 0.19-1.22	1.24		0.62-1.02
Glutamine				9.1	2.68-8.88

References: 1. Macy, Kelly and Sloan (1953)
2. Diem (1962)
3. Long (1961)

TABLE 13. COMPOSITION OF MILK AND SERUM. (b) VITAMINS AND MINERALS

Values in mg per 100 ml unless otherwise stated

Component	Bovine milk[1]	Bovine milk[2]	Bovine serum[3]	Human plasma[3]	Human plasma or serum[2]
Vitamins					
Biotin	3.5 µg	1.4-2.9 µg			0.95-1.66 µg
Choline Total	13.0	4-16	16.5		26-35
Free	4.0		4.0		0.3-1.5
Folic acid	0.23 µg	0.02-0.4 µg	2.0 µg	2.0µg	
m-Inositol	13	6-12		0.47	0.37-0.76
Nicotinic acid + nicotinamide	85 µg	50-86	1.21		
Pantothenic acid	350 µg	220-550 µg			6-22 µg
Para-aminobenzoic acid		0.015			
Pyridoxine	48 µg	40-63 µg			7 µg
Riboflavin	157 µg	116-202 µg	1.67 µg		0.3-1.3 µg
Thiamine	42 µg	28-90 µg			1.3 µg
Vitamin B_{12} (cyanocobalamin)	0.56 µg	0.32-1.24 µg			0.03-0.04 µg
Minerals					
Calcium	125	56-381	11.08		8-10.4
Chlorine	103	93-141	385		355-381
Iron		0.03-0.07	150-204 µg		0-0.2288
Magnesium	12	7-22	2.05		1.34-3.82
Manganese		3.3-3.6 µg	2.5-3.0µg		Up to 0.01
Phosphorus (total)	96				10.0-14.1
(inorganic)	80	56-112	5.56		2.56-4.16
Potassium	138	38-287	19.7		12.88-20.72
Sodium	58	39.2-139.2			31.0 -0.356
Copper		0.005-0.015			0.073-0.148

References: 1. Macy, Kelly and Sloan (1953)
2. Diem (1962)
3. Long (1961)

TABLE 13: COMPOSITION OF MILK AND SERUM.

(b) VITAMINS AND MINERALS

TABLE 13: COMPOSITION OF MILK AND SERUM (c) FATTY ACIDS AND MISCELLANEOUS COMPOUNDS

Values in mg per 100 ml unless otherwise stated.

Component	Bovine milk fat[4] (g per 100 g)	Bovine milk[2]	Bovine serum[3]	Human plasma or serum[2]
Fatty acids: saturated				
Butyric	4.0			
Caproic	2.3			
Caprylic	0.8			
Capric	1.9			
Lauric	2.2			
Myristic	9.3			
Palmitic	25.5			
Stearic	11.8			
As Arachidic	0.8			
Fatty acids: Unsaturated				
Decenoic	0.2			
Dodecenoic	0.2			
Tetradecenoic	0.9			
Hexadecenoic	2.3			
Oleic (octadecenoic	34.3			
Octadecadienoic	2.1			
Octadecatrienoic	-			
Eicosatetraenoic	1.4			
Others calculated as eicosadienoic acid				
Urea		as N 6.13-20.4	14.4-39.1	as N 9.6-17.6
Uric Acid		as N 1.13-3.69	0.9	2.3-6.9
Lactose		4.5-5 g		
Citric acid		5.65		1.6-3.2
Lactic acid		21.8		6.1-16.9
Cholesterol		(in plasma) 110		
Lecithin		68		
Sphingomyelin		29		
Cephalin		0-2		

References:

2. Diem (1962)
3. Long (1961)
4. Hilditch and Jasperson (1943) cited by Long (1961)

TABLE 13: COMPOSITION OF MILK AND SERUM

(c) FATTY ACIDS AND MISCELLANEOUS COMPOUNDS

Monod (1949) considered to be the simplest and best for the study of the growth of bacteria in batch cultures by the use of turbidity measurements.

Organisms growingin chemically defined media may differ phenotypically from organisms grown in empirical media or, more probably, from those growing in the natural habitat. Microscopic examinations were therefore carried out on the cultures in the defined media to detect the more obvious phenotypic morphological changes.

Methods and Results

Growth of the selected strains of C.bovis in defined media was compared with that obtained in a tryptose serum broth (TSB) which consisted of: tryptose (Oxoid), 10.0 g; Lab-Lemco beef extract, 3.0 g; sodium chloride, 5.0 g; distilled water, 1 litre. The pH was adjusted to 7.2 and, after sterilization, sterile bovine serum was added aseptically to a final concentration of 5% v/v.

The defined media and TSB were dispensed in 25 ml amounts in 100 ml Erlenmeyer flasks. During the course of the investigation it was found that the shaking to which the flasks were subjected during incubation allowed the amount of medium in each 100 ml flask to be increased to 50 ml without noticeably affecting the growth of the organisms. Since cotton wool closures can cause contamination of media with lipid material (Pollock, 1949; Hart et al., 1962), straight necked Erlenmeyer flasks fitted with stainless steel closures (Fisons Scientific Apparatus Ltd., Loughborough) were employed. The Erlenmeyer flasks and much of the other glassware used were of borosilicate glass.

All glassware except nephelometer tubes was cleaned by immersion for 24 hours in chromic acid (potassium dichromate, 63 g; distilled water, 35 ml; concentrated sulphuric acid, 960 ml) followed by at least six rinses with tap water and three rinses with glass-distilled water. Stainless steel flask closures and nephelometer tubes were cleaned by boiling for 10 minutes in a solution of Haemo-Sol (manufactured by Meinecke and Co., Inc., N.Y., and distributed by Alfred Cox (Surgical) Ltd., Coulsdon, Surrey), followed by at least six rinses with tap water

and three rinses with glass-distilled water.

In the preparation of the defined media pure nickel spatulae were used and these were flamed to red heat before use with each chemical. Constituents used in the preparation of media were of the purest grade manufactured by British Drug Houses Ltd., Poole, Dorset, except where stated otherwise. Supplements of vitamins (Supplements A, B and C) were prepared and sterilized following the recommendations of Hawk, Oser and Summerson (1947), and since adenine, guanine, uracil and choline chloride are heat stable (Hawk et al., 1947) they were added to the media before sterilization. After the distribution and sterilization of media, and the aseptic addition of sterile supplements, flasks of media were incubated at 37°C for 48 hours to detect any contamination.

Inocula

Corynebacterium bovis strains 124 and 147 were used throughout, Corynebacterium bovis strain 159 and C.bovis NCTC 3224 were also examined in certain media.

A three-day TSB culture was centrifuged and washed twice with sterile ¼-strength Ringer's solution (prepared with glass-distilled water). The twice-washed bacterial sediment was resuspended in sterile ¼-strength Ringer's solution, and the concentration of the suspension adjusted to give a turbidity reading of 25 on an EEL nephelometer (Evans Electroselenium Ltd., Halstead, Essex), the nephelometer having previously been adjusted to give readings of 0 and 100 with distilled

water and a Perspex turbidity standard respectively. Of this standard suspension, 0.025 ml was used to inoculate 25 ml of medium, or 0.05 ml was used to inoculate 50 ml of medium. A direct microscopic count of the suspensions indicated that the total number of bacteria introduced into 50 ml of medium was approximately 5×10^6.

Determination of growth

The amount of growth of the corynebacteria in the liquid media was determined nephelometrically by removing 2.5 ml of the culture at intervals during the period of incubation. The sample was diluted with 7.5 ml of glass-distilled water in a 6 x $\frac{5}{8}$ inch nephelometer tube. The turbidity was measured using the EEL nephelometer which had been adjusted to give readings of 0 and 100 with distilled water and an EEL Perspex standard respectively. In the event of the galvanometer deflection exceeding 100 turbidity units, further dilution of the cultures was carried out with glass-distilled water until the reading was within the galvanometer range.

A calibration curve of the turbidity of a number of dilutions of broth cultures indicated a relationship between turbidity and cell concentration which was linear up to a reading of about 85 units within the degree of consistency of the instrument. Thus the turbidity of the original broths could be calculated from the turbidity of the dilutions, provided that the dilution made caused the turbidity reading to be within the range 0-85 units. These values could then be corrected for the turbidity (if any) of the uninoculated medium, by substraction of the reading obtained with the uninoculated medium

(Meynell and Meynell, 1965). An uninoculated flask of each complete medium was incubated at 37°C and the turbidity of this medium determined each time the growth of the cultures was determined nephelometrically. This was necessary since in some cases (e.g. when lecithin was added to the medium without a solubilizer) the turbidity of the uninoculated medium increased during incubation. However, it was found that during the preliminary incubation at 37°C for 48 hours, which was used to detect contamination, the uninoculated media usually reached equilibrium with regard to their turbidity. The turbidity of cultures of each strain in TSB was used as a reference standard against which the suitability of the defined media could be determined.

The corrected turbidity readings thus obtained have been used for direct comparison of the growth rate and total growth of C.bovis in a variety of media. Since nephelometers are more sensitive for turbidity measurement than spectrophotometers or colorimeters, readings could be obtained with the normal 1:4 dilution used even when a very small amount of growth had occurred.

Nephelometer tubes and pipettes were cleaned with chromic acid or Haemo-Sol as already described in order to remove all grease and residues. Since the cultures were not incubated in the nephelometer tubes it was not possible to eliminate inaccuracies due to variability in tube diameter, thickness of the glass etc. by employing the same nephelometer tube that was always placed in the nephelometer with a constant orientation. Consequently inaccuracies due to tube variability were minimized by using optically standard tubes supplied

specifically for use with the EEL nephelometer, and examining the tubes for any tendency to asymmetric light scatter, surface marks, variations in thickness of glass or in diameter of the tube. As a result of such examination about 10% of the nephelometer tubes obtained were rejected on receipt.

During turbidity determinations the zero and full scale deflection were checked and adjusted frequently to correct for galvanometer drift, fatigue of the photo-electric cell etc. Photocell fatigue caused the readings to drift toward lower values, with the amount of drift increasing as the turbidity values increased, in contrast to the experience of Keily and Rogers (1955) who found that in the case of the Model 7 Coleman photonephelometer drift tended to be independent of the level of turbidity being measured.

Microscopic examination of the cultures in both the partially and fully defined media revealed that variations in the Gram reaction, and in the size and shape of the organisms frequently occurred. Therefore no attempt was made in this work to use the nephelometer readings to calculate bacterial numbers since, although nephelometer readings have been used to estimate cell concentrations (Lamanna and Mallette, 1953; Stárka and Koza, 1959), Powell (1963) noted that the Gram reaction, and the size and shape of the organisms had a profound effect upon the turbidity readings obtained. Of the morphological variations observed in the cultures of C.bovis, decrease in Gram positivity was likely to lead to a decrease in the turbidity of a given cell concentration, whereas decrease in size of the organisms probably resulted in greater

turbidity readings on the EEL nephelometer for a given cell concentration since the smaller the particle the greater the proportion of light scattered at large angles to the illuminating beam. Any attempt to correlate turbidity readings with cell concentration in nutritional studies was considered unrealistic because of the probable morphological variation.

The three apposite growth parameters for the determination of the suitability of a medium are duration of the lag phase, the rate of logarithmic growth and the total yield (Monod, 1949). Small inocula are well known to cause a longer lag phase than larger inocula. Also the inoculation of individual flasks of medium with a definite volume of a bacterial suspension can cause variations in the lag phase due to variations in the distribution of the test organism to the flasks although the total yield is less affected by such variations (Gavin, 1957). Unfortunately the complexity of the media from the point of view of the number of supplements that were added made it impracticable to inoculate the sterile basal medium in bulk followed by aseptic dispensation into sterile flasks - the only method of inoculation that can ensure an even distribution of the organism. If a small inoculum had been used with the method of inoculation employed, variation in distribution of the organism may have resulted in some flasks receiving an inoculum below the minimum number required to initiate growth. Consequently a comparatively large inoculum was used (see page 76), in order to minimize failures of growth in test media due to inoculum variation. In addition the use of a large inoculum reduced the

possibility of turbidity developing as a result of the growth of non-exacting mutants arising during the long lag phase which would obtain with a small inoculum.

Partially defined media

Partially defined medium 1

A number of amino acids were added in order to compensate for any deficiency in the casein hydrolysate.

The first medium examined had the following constitution:

Sodium chloride (AR grade)	7.0 g	Vitamin-free Casamino Acids (Difco)	5.0 g
Potassium dihydrogen phosphate (AR grade)	0.25 g	L-Arginine	20 mg
Dipotassium hydrogen phosphate	0.35 g	L-Cystine	20 mg
Ammonium dihydrogen phosphate (AR grade)	1.0 g	L-Histidine HCl	20 mg
Magnesium sulphate hydrated ($MgSO_4.7H_2O$)(AR grade)	0.25 g	DL-Methionine	10 mg
Copper sulphate, hydrated ($CuSO_4.5H_2O$)(AR grade)	0.003 g	DL-Serine	20 mg
Ferrous sulphate, hydrated ($FeSO_4.7H_2O$)(AR grade)	0.007 g	DL-Threonine	20 mg
D-glucose (AR grade)	1.0 g	DL-Tryptophan	30 mg
Sodium acetate, hydrated ($CH_3COONa.3H_2O$)(AR grade)	0.1 g	Adenine	10 mg
Glass-distilled water	to 1 litre	Guanine	10 mg

The ingredients were added individually at room temperature to 750 ml of glass-distilled water which was continuously agitated using a magnetic stirrer. Each ingredient was allowed to dissolve or disperse thoroughly before the next was added. The L-cystine and guanine were added to the minimum amount of 20% (w/v) sodium hydroxide (AR grade reagent dissolved in glass-distilled water) required to obtain complete solution of these two compounds. This solution was

then added to, and mixed with, the water containing the rest of the ingredients. The volume was then made up to 1 litre with glass-distilled water. The mouth of the flask containing the medium was covered with a glass beaker and the medium was autoclaved at 107°C for 5 minutes. After the medium had cooled, the pH was adjusted to 7.2 (determined by a Pye Universal pH meter with a Pye/Ingold combined glass/reference electrode and automatic temperature compensation by means of a resistance thermometer) using N sodium hydroxide and N hydrochloric acid.

After adjustment of the pH, the medium was clarified through an asbestos filter (Carlson filter Grade K5) and distributed in 25 ml amounts in 100 ml Erlenmeyer flasks fitted with stainless steel closures. The flasks of medium were sterilized by autoclaving at 115°C for 20 minutes.

Growth of C.bovis strains 124 and 147 in the above medium was determined without further supplementation and with the addition of supplement A; supplement B; supplements A and B; supplements A, B and C; or supplements A, B, C and additional glucose.

Supplement A: riboflavin, 2 mg; thiamin HCl, 2 mg; dissolved in 100 ml of 0.02 N acetic acid (AR grade). Sterilized by filtration and stored in the refrigerator. This was added to the media at a rate of 0.025 ml per 25 ml of medium.

Supplement B: calcium pantothenate, 2 mg; nicotinic acid, 20 mg; folic acid, 200 µg; pyridoxal HCl, 6 mg; p-aminobenzoic acid, 2 mg; dissolved in 100 ml of glass-distilled water. Sterilized by filtration

and stored in the refrigerator. This was added to the media at a rate of 0.25 ml per 25 ml of medium.

Supplement C: pyridoxine HCl, 10 mg, dissolved in 100 ml glass-distilled water, sterilized by filtration and stored in the refrigerator. This was added to the media at a rate of 0.025 ml per 25 ml of medium.

Glucose was added as a filter-sterilized 10% (w/v) solution, to increase the final concentration of glucose in the medium to 0.11%.

Results

No growth was obtained in any of the media.

Partially defined medium 2 (PDM 2)

In a further series of media the partially defined medium 1 (PDM1) with the addition of supplements A, B and C was adopted as the basal medium (PDM 2).

Amongst the supplements whose effect upon growth was studied in this series were pimelic acid and β-alanine, substances shown by Mueller to be either required by, or stimulatory for, C.diphtheriae (Mueller, 1937; Mueller and Cohen, 1937); m-inositol, known to be required by a number of micro-organisms (see Florkin and Mason, 1962); and biotin, a vitamin involved in the synthesis of unsaturated fatty acids and shown by Williams and co-workers (1947) to be interchangeable with oleic acid in media used for the growth of Lactobacillus spp.

The media used were the following:

PDM 2-1 : no further supplementation

PDM 2-2 : + supplement D

PDM 2-3 : + m-inositol

PDM 2-4 : + biotin

PDM 2-5 : + supplement D and biotin

PDM 2-6 : + supplement D; m-inositol; and biotin

PDM 2-7 : + supplement D; m-inositol; biotin; and sodium lactate.

PDM 2-8 : + supplement D; biotin; and yeast extract

PDM 2-9 : + supplement D; m-inositol; biotin; sodium lactate; and albumin

PDM 2-10 : + supplement D; m-inositol; biotin; sodium lactate; yeast extract; albumin; and glucose

Supplement D: pimelic acid, 15 mg; β-alanine 23 mg; dissolved in 100 ml of 0.02 N acetic acid (AR grade) sterilized either by autoclaving for 20 minutes at 115°C or by filtration, and stored in the refrigerator. This was added to media at a rate of 0.025 ml per 25 ml of medium.

m-Inositol: 0.05 g of m-inositol in 100 ml of 0.02 N acetic acid (AR grade), sterilized by filtration and stored in the refrigerator; 0.25 ml added to each 25 ml of medium.

Biotin: 0.25 ml of sterile biotin solution (containing 1 μg biotin per ml in phosphate buffer, obtained from British Drug Houses Ltd., Poole, Dorset) was added to 25 ml of medium to give a final concentration of biotin of 10 μg/litre.

Sodium lactate: sodium lactate solution (B.D.H.), 7.1 ml; distilled water, 17.9 ml; sterilized by filtration and 0.025 ml added to 25 ml of medium.

Yeast extract: yeast extract (Oxoid), 5 g; made up to 100 ml of

solution with distilled water and sterilized by filtration; 0.25 ml added to 25 ml of medium.

Albumin: bovine serum albumin, fraction V (Armour Pharmaceuticals Co. Ltd., Eastbourne) 3% (w/v) in distilled water and sterilized by filtration; 0.25 ml added to 25 ml of medium.

Glucose: 0.25 ml of a filter-sterilized 20% (w/v) solution of D-glucose (AR grade) added to 25 ml of medium.

Results

An extremely small amount of growth of C.bovis strains 124 and 147 occurred in PDM 2 - 8 and PDM 2 - 10, the only two media with yeast extract included. Microscopic examination revealed that the culture of C.bovis strain 147 consisted mostly of coccal cells that stained Gram-negative. The culture of C.bovis strain 124 also consisted of atypical organisms which were either coccal or very long and thread-like, staining in Gram-positive patches with colourless patches giving a heavily barred appearance.

Partially defined medium 3 (PDM 3)

This medium was similar in content to PDM 2 except for the following alterations in concentration: ammonium dihydrogen phosphate 0.5 g/l, DL-threonine, 30 mg/l; and the addition of choline chloride, 10 mg, and uracil, 10 mg, to each litre of medium before sterilization. Supplements A, B and C were added as before.

Amongst the supplements whose effect upon growth was studied in this series were thioctic acid (or α-lipoic acid, a substance found by Stokstad, Hoffmann and Belt (1950) to be necessary for the growth

of a Corynebacterium) and ovolecithin (C.bovis having been found capable of growing on egg-yolk agar).

The growth of C.bovis strains 124 and 147 was determined in this medium without further supplementation (PDM 3-1) and after the following additions.

PDM 3-2 : + biotin (see above) and DL-thioctic acid

PDM 3-3 : + biotin; m-inositol (see above); and DL-thioctic acid

PDM 3-4 : + supplement D (see above); DL-thioctic acid and ovolecithin

PDM 3-5 : + supplement D; DL-thioctic acid and glycerol

PDM 3-6 : + supplement D; DL-thioctic acid; ovolecithin; glycerol; yeast extract (see above); and casein extracts.

DL-thioctic acid: DL-thioctic acid (DL - α-lipoic acid)(British Drug Houses Ltd.), 1 mg, dissolved in 100 ml of distilled water sterilized by filtration and stored in a refrigerator; 0.025 ml added aseptically to 25 ml of medium to give a final concentration of 10 μg per litre.

Ovolecithin: 95-100% ovolecithin (British Drug Houses Ltd.), 80 mg, dissolved in 10 ml of absolute ethanol (AR grade), sterilized by filtration and stored in a refrigerator; 0.25 ml added to 25 ml of medium to give a final concentration of 80 mg lecithin per litre of medium.

Glycerol: sterilized by autoclaving at 115°C for 20 minutes.

Casein extracts: an aqueous extraction of fat-free, vitamin-free casein (Difco) was made by suspending 5 g of the casein in 100 ml of distilled water, after which the suspension was centrifuged and the aqueous supernatant liquid removed and sterilized by filtration. An ethanolic extraction of fat-free, vitamin-free casein was made in a

similar manner by suspending 5 g of the casein in 100 ml of absolute ethanol, followed by centrifugation, and sterilization by filtration of the ethanolic supernatant liquid. These extracts were added to media at the rate of 0.2 ml of each per 25 ml of medium.

Results

Good growth of C.bovis strains 124 and 147 was obtained in the media PDM 3-4, and PDM 3-6 which were the two media that included ovolecithin. No growth occurred in the other media. It appeared therefore that C.bovis had a nutritional requirement that was satisfied by lecithin or by a growth factor present as an impurity in the ovolecithin.

Partially defined medium 4 (PDM 4)

The concentrations of amino acids and certain other constituents in this medium were increased, the medium having the following constitution:

Sodium chloride (AR grade)	7.0 g	Vitamin-free Casamino Acids (Difco)	7.5 g
Potassium dihydrogen phosphate (AR grade)	0.25 g		
Dipotassium hydrogen phosphate	0.35 g	L-Arginine	200 mg
Ammonium dihydrogen phosphate (AR grade)	0.5 g	L-Cystine	200 mg
Magnesium sulphate, hydrated ($MgSO_4.7H_2O$)(AR grade)	0.25 g	L-Histidine HCl	200 mg
Copper sulphate, hydrated ($CuSO_4.5H_2O$)(AR grade)	0.003g	DL-Methionine	100 mg
Ferrous sulphate, hydrated ($FeSO_4.7H_2O$)(AR grade)	0.007g	DL-Serine	200 mg
D-Glucose (AR grade)	1.0 g	DL-Threonine	300 mg
Sodium acetate, hydrated ($CH_3COONa.3H_2O$)(AR grade)	0.3 g	DL-Tryptophan	300 mg
Distilled water	to 1 litre	Adenine	15 mg
		Uracil	15 mg
		Choline chloride	15 mg
		Guanine	15 mg

The method of preparation was as described for PDM 1. The Supplements A, B and C, and sodium oleate were added to the basal medium after sterilization.

Sodium oleate solution (Cohen, 1949): 0.1 ml of oleic acid (British Drug Houses Ltd.) and 0.1 ml of 40% (w/v) sodium hydroxide were mixed together with a glass rod. After thorough mixing 9.9 ml of distilled water were added and the sodium oleate dissolved by heating. The solution was sterilized by autoclaving at 115°C for 20 minutes. To each 25 ml of medium 0.025 ml of solution was added aseptically, giving a final concentration of sodium oleate of about 1 in 100,000.

The growth of C.bovis strains 124 and 147 was determined without further supplementation (PDM 4-1) and after the following additions.

PDM 4-2 : + biotin and DL-thioctic acid

PDM 4-3 : + biotin; m-inositol; and DL-thioctic acid

PDM 4-4 : + supplement D; DL-thioctic acid, and ovolecithin

PDM 4-5 : + supplement D; DL-thioctic acid; and glycerol

PDM 4-6 : + supplement D; DL-thioctic acid; ovolecithin; glycerol; yeast extract; and both casein extracts

PDM 4-7 : + supplement D and m-inositol

PDM 4-8 : + supplement D; DL-thioctic acid, and the aqueous extract of casein.

Results

Good growth of C.bovis strains 124 and 147 was obtained in the media PDM 4-4 and PDM 4-6, which were the two media that included ovolecithin. The total amount of growth produced in each was almost identical with that produced in PDM 3-4 and PDM 3-6 respectively, but

there were lower growth rates in the media PDM 4-4 and PDM 4-6. Consequently, further development of the partially defined media was based on PDM 3, and since the addition of ovolecithin had resulted in good growth, the combinations of supplements chosen for study were designed to investigate this requirement. During this stage of the experiment the four supplements A, B, C and D were added to the basal medium, without endeavouring to define the vitamin requirements of C.bovis.

Partially defined medium 5 (PDM 5)

The basal medium was the same as PDM 3, with the addition of the four supplements A, B, C and D.

The growth of C.bovis strains 124 and 147 in this medium was determined after the following additions.

	Ovolecithin	DL-thioctic acid	Glycerol	Yeast extract	Sodium oleate	Aqueous extract of casein	Ethanolic extract of casein
PDM 5-1							
PDM 5-2	+	+					
PDM 5-3	+						
PDM 5-4		+					
PDM 5-5		+	+				
PDM 5-6			+				
PDM 5-7			+	+			
PDM 5-8	+	+			+		
PDM 5-9		+	+		+		
PDM 5-10	+	+	+	+		+	+
PDM 5-11	+	+	+	+			
PDM 5-12	+			+			

C.bovis strain 124 was inoculated into PDM 5-1, PDM 5-2, PDM 5-3, PDM 5-4, PDM 5-5, PDM 5-6, PDM 5-7, PDM 5-8 and PDM 5-9. C.bovis strain 147 was inoculated into PDM 5-1, PDM 5-2, PDM 5-3, PDM 5-5,

PDM 5-6, PDM 5-7, PDM 5-8, PDM 5-9, PDM 5-10, PDM 5-11, and PDM 5-12.

Results

The turbidity readings of the cultures are shown in Table 14, and selected growth curves in Figure 1. The "corrected turbidity units" (C.T.U.) consisted of the nepholometer readings obtained with the diluted cultures corrected both for the dilution that was carried out before the nephelometric determinations and for the turbidity of the uninoculated medium. Negligible growth of C.bovis strain 124 was obtained in PDM 5-1, PDM 5-4, PDM 5-5, PDM 5-6, PDM 5-7, and no growth was obtained in PDM 5-9. Negligible growth of C.bovis strain 147 was obtained in PDM 5-1, PDM 5-5, PDM 5-6, PDM 5-7 and no growth occurred in PDM 5-9. Thus, the growth-stimulating properties of ovolecithin were confirmed. This requirement for lecithin was not capable of being replaced by sodium oleate, but it was possible that the sodium oleate was actually inhibitory to the organisms, the inhibitory effect being removed by the addition of lecithin (Kodicek and Worden, 1945; Kodicek, 1949). Growth of the two strains in PDM 5-3 indicated that thioctic acid (α-lipoic acid) was not required for growth, although C.bovis appeared to be slightly stimulated by its presence.

Further series using PDM 5

Since the ovolecithin, although of 99-100% purity, was a natural lecithin extracted from egg-yolk, stimulation of growth of C.bovis could have been due to one or more of the many other substances present in the preparation as traces. Consequently in this next series of media, ovolecithin was replaced by synthetic lecithins (synthetic

TABLE 14. GROWTH OF C.BOVIS STRAINS 124 AND 147 IN MEDIUM PDM 5

Medium and organism	Growth of C.bovis, in C.T.U. after incubation for the following times (hours)					Microscopic appearance
	28 h	71 h	98 h	172 h	216 h	
C.bovis strain 124						
PDM 5-2	2	70	82	96	120	Typical
PDM 5-3	1	68	80	96	106	Typical
PDM 5-8	-	34	68	108	126	Typical
TSB	14	33	71	130	157	Typical
C.bovis strain 147						
PDM 5-2	0	74	98	95	100	Typical
PDM 5-3	0	60	72	73	85	Typical
PDM 5-8	-	95	110	120	124	Typical
PDM 5-10	22	102	102	104	112	Thin rods palely staining
PDM 5-11	15	101	100	100	108	Typical
PDM 5-12	2	100	115	116	116	Typical
TSB	4	66	81	114	122	Typical

FIGURE 1.

Figure 1. Growth of C.bovis strains 147 and 124 in partially defined medium 5

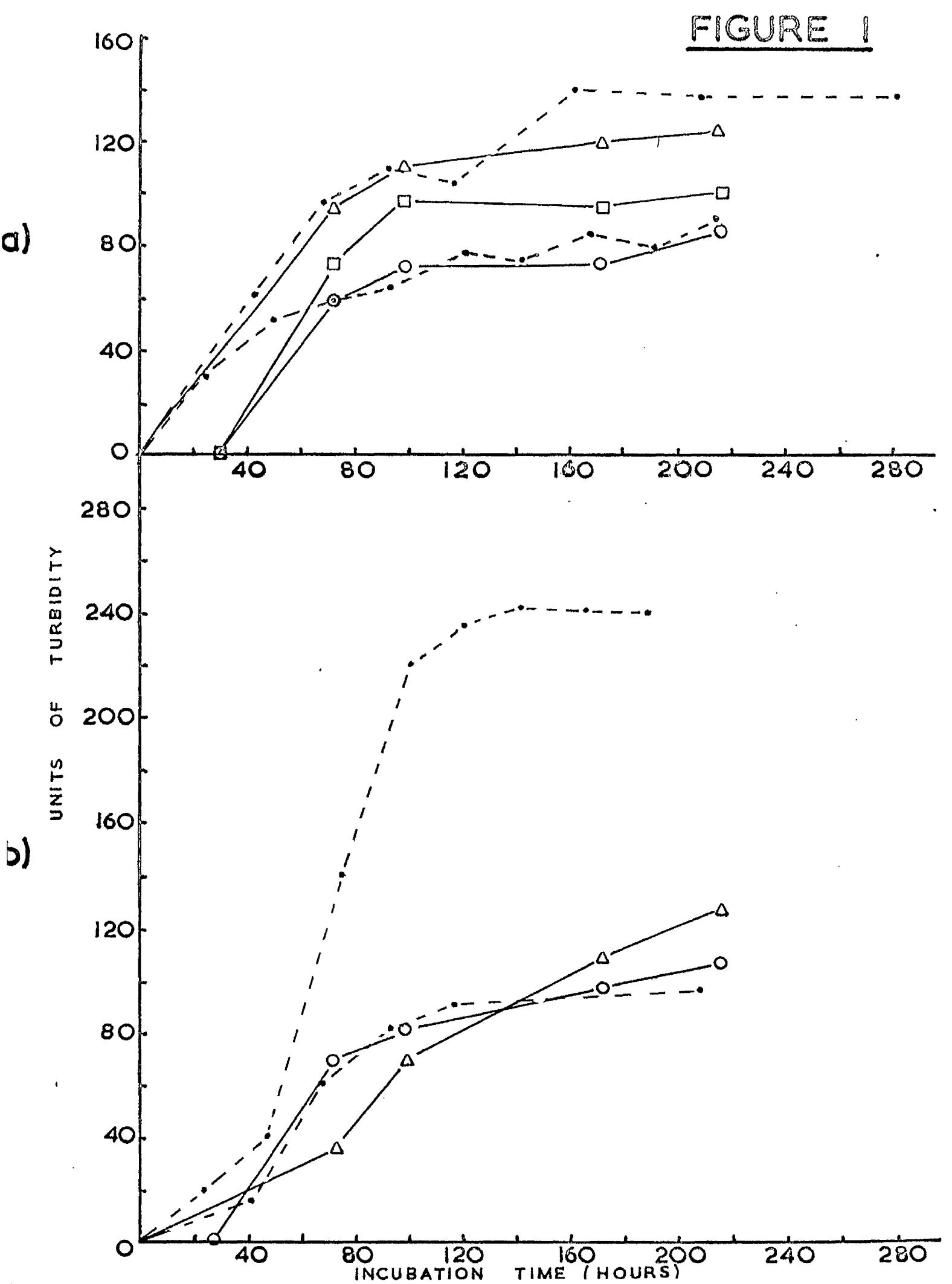
a) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 147 in PDM 5.

b) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 124 in PDM 5.

Open circles, 80 mg of ovolecithin added per litre of PDM 5 (PDM 5-3).
Open squares, 80 mg of ovolecithin and 10 µg of DL-thioctic acid added per litre of PDM 5 (PDM 5-2)
Open triangles, 80 mg of ovolecithin, 10 µg of DL-thioctic acid and 10 mg of sodium oleate added per litre of PDM 5 (PDM 5-8)

Broken lines indicate the minimum and maximum growths in tryptose serum broth containing different batches of serum (TSB).

FIGURE 1


a)
b)
160
120
80
40
0
280
240
200
160
120
80
40
0
40
80
120
160
200
240
280
UNITS OF TURBIDITY
INCUBATION TIME (HOURS)

β,γ -dipalmitoyl L- α -phosphatidylcholine, or the racemic form).

The basal medium was the same as PDM 3, with the addition of the four supplements A, B, C and D. The growth of C.bovis strains 124 and 147 in this medium was determined after supplementation as indicated below.

PDM 5-13 : + DL-thioctic acid and sodium oleate

PDM 5-14 : + DL-thioctic acid and ovolecithin (4 mg/l)

PDM 5-15 : + DL -thioctic acid and L- α -lecithin (80 mg/l)

PDM 5-16 : + DL-thioctic acid and L- α -lecithin (8 mg/l)

PDM 5-17 : + DL-thioctic acid and sodium β -glycerophosphate (40 mg/l)

PDM 5-18 : + DL-thioctic acid and sodium β-glycerophosphate (4 mg/l)

PDM 5-19 : + DL-thioctic acid and glycerophosphoric acid (40 mg/l)

PDM 5-20 : + DL-thioctic acid and glycerophosphoric acid (4 mg/l)

PDM 5-21 : + DL-thioctic acid and ethanolamine phosphoric acid (40 mg/l)

PDM 5-22 : + DL-thioctic acid and ethanolamine phosphoric acid (4 mg/l)

PDM 5-23 : + DL-thioctic acid and DL- α -lecithin (80 mg/l)

PDM 5-24 : + DL-thioctic acid and DL- α -lecithin (8 mg/l)

L- α -lecithin: 80 mg of β,γ -dipalmitoyl-L- α -lecithin (synthetic, puriss, chromatographically pure, manufactured by Fluka AG, Buchs SG, Switzerland), were dissolved in 10 ml absolute ethanol (AR grade) and sterilized by filtration. To obtain a nominal concentration of 80 mg lecithin per litre of medium, 0.25 ml of solution was added to 25 ml of medium. During the addition of the solution, the tip of the pipette was held beneath the surface of the medium which was swirled, in order to obtain rapid dispersion of the lecithin.

Sodium β-glycerophosphate: sodium β-glycerophosphate (British Drug Houses Ltd.), 400 mg, were dissolved in 100 ml glass-distilled water and sterilized by filtration. To obtain a nominal concentration of 40 mg per litre of medium, 0.25 ml of solution was added to 25 ml of medium.

Glycerophosphoric acid: glycerophosphoric acid (British Drug Houses Ltd.), 400 mg, were dissolved in 100 ml of glass-distilled water and sterilized by filtration. To obtain a nominal concentration of 40 mg of glycerophosphoric acid per litre of medium, 0.25 ml of solution was added to 25 ml of medium.

Ethanolamine phosphoric acid: ethanolamine phosphoric acid (British Drug Houses Ltd.), 400 mg, were dissolved in 100 ml of glass-distilled water and sterilized by filtration; 0.25 ml was added to 25 ml of medium to obtain a nominal concentration of 40 mg/l.

DL- α-lecithin: 80 mg of β, γ -dipalmitoyl DL- α -lecithin, (synthetic, purum) (manufactured by Fluka AG, Buchs SG, Switzerland) were dissolved in 10 ml of absolute ethanol (AR grade) and sterilized by filtration. To obtain a nominal concentration of 80 mg of lecithin per litre of medium, 0.25 ml of solution was added to 25 ml of medium.

Results

The turbidity of the cultures in Corrected Turbidity Units (CTU) are shown in Table 15, and selected growth curves are shown in Figure 2.

The substitution of synthetic lecithin for ovolecithin supported the growth of both strains, although less growth was obtained. Synthetic DL- α -lecithin resulted in slightly greater total growth

TABLE 15. GROWTH OF C.BOVIS STRAINS 124 AND 147 IN MEDIUM PDM 5

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)								Microscopic appearance
	22h	43h	69h	93h	117h	163h	209h	282h	
C.bovis strain 124									
PDM 5-2	0	2	64	97	110	136	140	143	Typical
PDM 5-13	0	0	2	1	2	4	1	0	
PDM 5-14	0	0	2	2	0	3	1	1	
PDM 5-15	0	2	34	40	38	36	56	54	Typical
PDM 5-16	0	0	0	0	-	-	-	-	
PDM 5-17	0	0	2	3	1	3	-	-	
PDM 5-18	0	0	1	41	66	86	99	86	Abnormal in morphology and staining.
PDM 5-19	0	0	31	53	70	69	69	70	Extremely short rods, Gram-positive
PDM 5-20	0	0	1	50	65	72	71	73	Abnormal in morphology and staining
PDM 5-21	0	2	76	77	76	79	79	79	Extremely short rods, Gram-positive
PDM 5-22	0	0	0	0	0	0	0	0	
PDM 5-23	5	22	41	40	43	52	60	60	Typical
PDM 5-24	0	0	0	7	91	103	105	105	Abnormal in morphology and staining
TSB	-	15	60	80	90	-	96	-	Typical
C.bovis strain 147									
PDM 5-13	0	0	2	1	2	1	1	0	
PDM 5-14	0	0	2	2	1	2	1	3	
PDM 5-15	2	20	35	38	40	37	38	40	Typical
PDM 5-16	0	0	0	0	-	-	-	-	
PDM 5-17	0	0	0	2	2	1	-	-	
PDM 5-18	0	0	1	2	0	1	1	0	
PDM 5-19	0	0	0	0	0	0	0	0	
PDM 5-20	0	0	0	0	0	0	1	0	
PDM 5-21	0	0	1	1	1	1	0	2	
PDM 5-22	0	0	1	1	2	1	1	0	
PDM 5-23	16	30	42	39	39	45	60	59	Typical
PDM 5-24	1	0	0	0	0	0	0	0	
TSB	-	62	98	110	105	140	138	138	Typical

FIGURE 2

Figure 2. Growth of C.bovis strains 147 and 124 in partially defined medium 5

a) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 147 in PDM 5.

b) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 124 in PDM 5.

Open triangles, 80 mg of ovolecithin and 10 µg of DL-thioctic acid added per litre of PDM 5 (PDM 5-2)

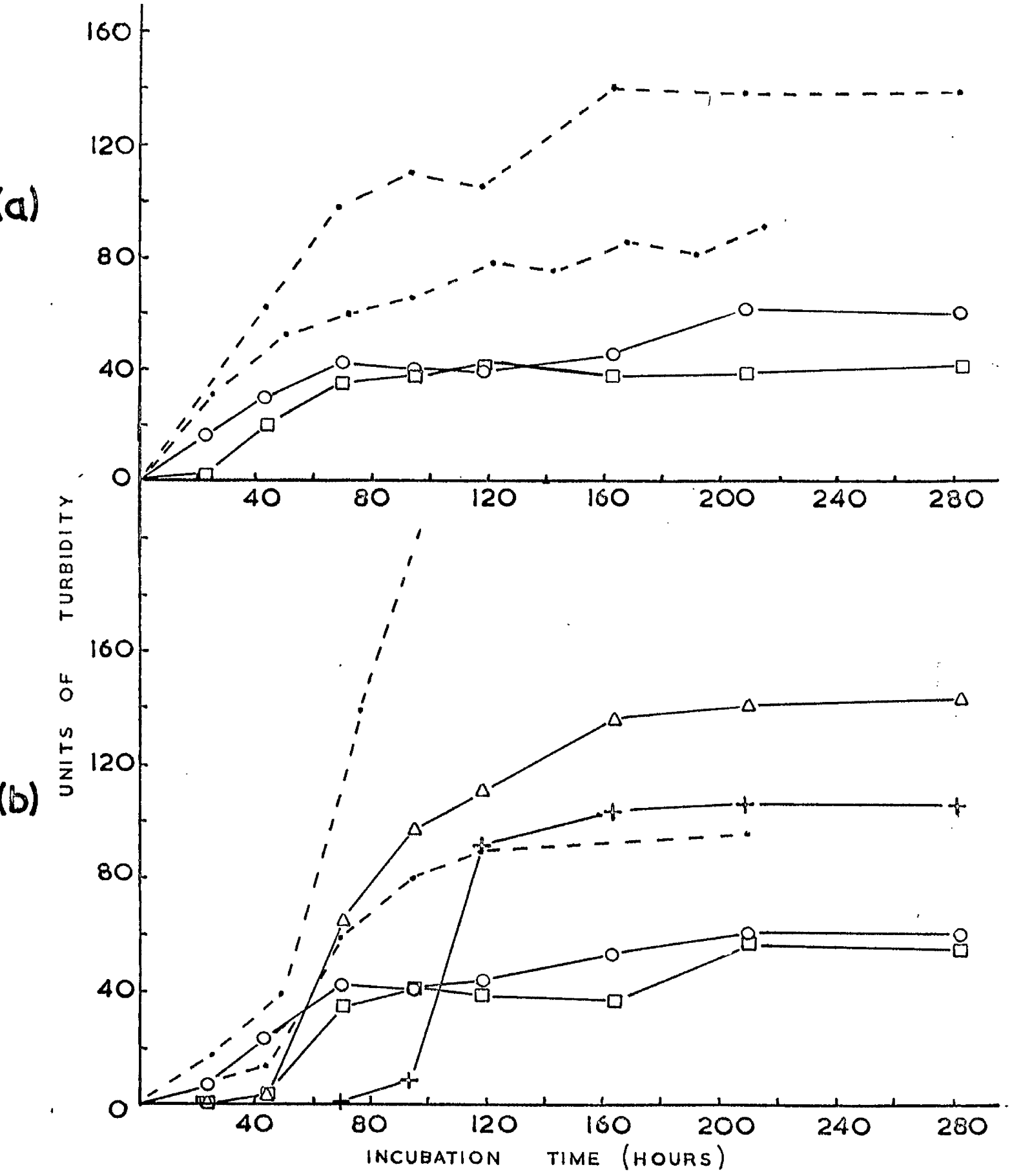
Open squares, 80 mg of synthetic L-α-lecithin and 10 µg of DL-thioctic acid added per litre of PDM 5 (PDM 5-15)

Open circles, 80 mg of synthetic DL-α-lecithin and 10 µg of DL-thioctic acid added per litre of PDM 5 (PDM 5-23)

Plus signs, 8 mg of synthetic DL-α-lecithin and 10 µg of DL-thioctic acid added per litre of PDM 5 (PDM 5-24).

Broken lines indicate the minimum and maximum growths in tryptose serum broth containing different batches of serum (TSB).

FIGURE 2


(a)
(b)
160
120
80
40
0
40
80
120
160
200
240
280
UNITS OF TURBIDITY
INCUBATION TIME (HOURS)

than was obtained with synthetic L- α-lecithin. Growth was obtained when ovolecithin or synthetic lecithin was present in a concentration of 80 mg/l but no growth occurred when the concentration was reduced to 8 mg/l. That the growth response was not due to the addition of the ethanol used as the solvent for the lecithin, was demonstrated by the absence of growth of the two strains in PDM 5 to which the appropriate amount of absolute ethanol had been added with the thioctic acid. In the case of C.bovis strain 124 substantial growth was obtained in the media containing 4 mg sodium β-glycerophosphate/l (PDM 5-18), glycerophosphoric acid (PDM 5-19 and PDM 5-20) and 40 mg ethanolamine phosphoric acid/l (PDM 5-21). In the case of the media in which sodium β-glycerophosphate or glycerophosphoric acid was included at a concentration of 4 mg/l, the growth obtained was grossly abnormal both in its morphology and in the reaction to Gram's stain. In the media containing the higher concentration of glycerophosphoric acid or ethanolamine phosphoric acid the organisms, although staining Gram-positive, were atypical in that they were predominantly extremely short rods, of even diameter with the usual club-shapes and swollen ended rods being absent.

In spite of the slightly higher growth rate and increase in yield obtained when DL- α-lecithin was used rather than L- α-lecithin, in subsequent experiments L- α-lecithin was the synthetic lecithin chiefly used, firstly because of the greater purity of the L- α-lecithin and secondly because of the greater availability of the latter.

Fully defined media

The results obtained from the use of the partially defined medium PDM 5 in which the two organisms C.bovis strains 124 and 147 were able to grow when synthetic lecithin was added to the medium suggested that at this stage it should be possible to formulate a fully defined medium with the vitamin-free Casamino acids of the partially defined media being replaced by named amino acids. The distribution in proteins of amino acids and their inclusion in typical defined media is shown in Table 16.

In the medium identification numbers used in this section the first numeral following the letters "FDM" indicates the basal medium used, and the suffixed number refers to a particular type of supplementation which is consistent throughout the series. Thus FDM 2-4, FDM 3-4, FDM 4-4 all contain 80 mg ovolecithin per litre of basal medium.

Fully defined medium 1 (FDM 1)

In the first medium used (FDM 1) the vitamin-free Casamino acids were replaced by only eleven amino acids, subsequent media being formulated with increasing numbers of amino acids. In addition to inorganic salts and amino acids, this medium also included the purines and pyrimidine, adenine, guanine, and uracil which are known to be required by a number of bacteria, including C.diphtheriae, which was shown by Dalby and Holdsworth (1956) to require adenine + guanine (or adenine + xanthine, or hypoxanthine); and sodium acetate, which acts as a growth stimulant for many bacteria (Snell, 1961).

TABLE 16: NATURAL DISTRIBUTION OF AMINO ACIDS AND THEIR USE IN DEFINED MEDIA

Amino Acid	OCCURRENCE			USE IN DEFINED MEDIA			
	Commonly found in proteins	Reported in serum or plasma	Reported in bovine milk	Required by bacteria	Suggested by Meynell & Meynell (1965)	Used by Chattaway et al (1949)	Used in tissue culture media (Paul 1965)
Alanine	+	+		+	+	+	+
α-Aminobutyric acid		+					+
Arginine	+	+	+	+	+	+	+
Asparagine	(+)	+		+	+		+
Aspartic acid	+	+		+	+	+	+
Citrulline		+					
Creatine		+					
Creatinine		+					
Cysteine	+						+
Cystine	+	+		+	+	+	+
Glucosamine							+
Glutamic acid	+	+		+	+	+	+
Glutamine	(+)	+		+	+		+
Glycine	+	+		+	+	+	+
Histidine	+	+	+	+	+	+	+
Hydroxyproline	(+)			+	+		+
Isoleucine	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+
Lysine	+	+	+	+	+	+	+
Methionine	+	+	+	+	+	+	+
Ornithine	(+)	+					+
Phenylalanine	+	+	+	+	+	+	+
Proline	+	+		+	+	+	+
Serine	+	+		+	+	+	+
Taurine		+					+
Threonine	(+)	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+	+
Tyrosine	+	+		+	+	+	+
Valine	+	+	+	+	+	+	+

Vitamins and growth factors were added in the form of the supplements A, B, C and D previously used.

This medium had the following constitution:

Sodium chloride (AR grade)	5.0 g	DL-α-Alanine	0.05 g
Potassium dihydrogen phosphate (AR grade)	0.25 g		
Dipotassium hydrogen phosphate	0.35 g	L-Arginine HCl	0.05 g
Ammonium dihydrogen phosphate (AR grade)	0.5 g	L-Asparagine	0.05 g
Magnesium sulphate, hydrated ($MgSO_4.7H_2O$)(AR grade)	0.25 g	Aspartic acid	0.05 g
Copper sulphate, hydrated ($CuSO_4.5H_2O$)(AR grade)	0.003g	L-Cystine	0.05 g
Ferrous sulphate, hydrated ($FeSO_4.7H_2O$)(AR grade)	0.007g	L-Cysteine HCl	0.05 g
D-Glucose (AR grade)	1.0 g	L-Glutamic acid	0.05 g
Sodium acetate,hydrated ($CH_3COONa.3H_2O$)(AR grade)	0.1 g	Glycine (AR grade)	0.05 g
		DL-Leucine	0.05 g
Glass-distilled water	to 1 litre	L-Lysine HCl	0.05 g
		L-Proline	0.05 g
		Adenine	0.01 g
		Guanine	0.01 g
		Uracil	0.01 g
		Choline chloride	0.01 g

The inclusion of cysteine in addition to cystine was probably unnecessary since cysteine is oxidized in neutral or alkaline solutions by atmospheric oxygen to cystine and the latter is readily converted to cysteine by most organisms.

The method of preparation was the same as that described for PDM 1, with the L-cystine and guanine being dissolved in a small amount of 20% (w/v) of sodium hydroxide (AR grade reagent) which was then added to the water, as before. After solution of the ingredients, the medium was autoclaved for 5 minutes at 108°C, cooled and the reaction adjusted to pH 7.2. The medium was then clarified by filtration through a Grade K5 Carlson-Ford asbestos filter, distributed in 25 ml amounts in 100 ml

Erlenmeyer flasks fitted with stainless steel closures, and sterilized by autoclaving at 115°C for 20 minutes.

After sterilization the vitamin and growth factor supplements A, B, C and D were added aseptically. Growth of C.bovis strain 124 was determined in this medium without further supplementation (FDM 1-1) and after supplementation as indicated below.

FDM 1-2 : + L-α-lecithin (80 mg/l)

FDM 1-3 : + DL-α-lecithin (80 mg/l)

FDM 1-4 : + ovolecithin (80 mg/l)

FDM 1-5 : + sodium β-glycerophosphate (40 mg/l)

FDM 1-6 : + glycerophosphoric acid (40 mg/l)

FDM 1-7 : + ethanolamine phosphoric acid (40 mg/l)

FDM 1-8 : + Tween 80 (5 ml/l)

FDM 1-11x: + Tween 80 (5 ml/l) and L-α-lecithin (80 mg/l)

FDM 1-12x: + Tween 80 (5 ml/l) and DL-α-lecithin (80 mg/l)

Tween 80: Polyoxyethylene sorbitan monooleate (Tween 80, manufactured by Atlas Chemical Industries Inc., Delaware and distributed by Honeywill and Stein Ltd., Carshalton, Surrey).

Results

No growth of C.bovis strain 124 occurred in any of the media.

Fully defined medium 2 (FDM 2)

This medium was similar to FDM 1, but with the addition of L-histidine HCl, DL-methionine, DL-serine, DL-threonine and DL-tryptophan, which were added to the medium in the initial stages of preparation, each at a concentration of 0.05 g per litre of medium.

Vitamin and growth factor supplements A, B, C and D were added after sterilization as before.

The growth of C.bovis strains 124 and 147 was determined in this medium without further supplementation (FDM 2-1) and after supplementation as indicated below.

FDM 2-2 : + L-α-lecithin (80 mg/l)

FDM 2-3 : + DL-α-lecithin (80 mg/l)

FDM 2-4 : + ovolecithin (80 mg/l)

FDM 2-5 : + sodium β-glycerophosphate (40 mg/l)

FDM 2-6 : + glycerophosphoric acid (40 mg/l)

FDM 2-7 : + ethanolamine phosphoric acid (40 mg/l)

Results

The turbidities of the cultures in Corrected Turbidity Units (CTU) are shown in Table 17 and selected growth curves are shown in Figure 3. Growth occurred in the media containing natural lecithin and slight growth occurred in the media containing synthetic lecithin.

The growth obtained in the media containing synthetic lecithins was considerably less than that obtained in the equivalent partially defined media (PDM 5) containing the appropriate lecithins. Apart from the probability that the basal medium was not as suitable as PDM 5 it was noted during the preparation of FDM 2-2 and FDM 2-3 that neither of the synthetic lecithins gave as fine a dispersion as that obtained when it was added to the partially defined medium, some particles being readily visible to the naked eye by oblique lighting. The natural ovolecithin dispersed more readily, probably due partly

TABLE 17 GROWTH OF C.BOVIS STRAINS 124 AND 147

IN MEDIUM FDM 2

Medium and organism	Growth of C.bovis, in CTU, after incubation for the following times (hours)									Microscopic appearance
	24h	48h	76h	101h	121h	142h	166h	190h		
C.bovis strain 124										
FDM 2-1	0	0	0	0	0	0	0	0		
FDM 2-2	0	0	2	4	4	6	4	4		
FDM 2-3	0	0	0	2	2	2	0	0		
FDM 2-4	0	6	10	12	14	12	14	15		Typical
FDM 2-5	0	0	0	0	0	0	0	0		
FDM 2-6	0	0	0	0	0	0	0	0		
FDM 2-7	0	0	0	0	0	0	0	0		
TSB	18	40	140	220	235	242	240	240		
	24h	50h	72h	94h	121h	142h	168h	192h	215h	
C.bovis strain 147										
FDM 2-1	0	0	0	0	0	0	0	0	0	
FDM 2-2	0	12		10	12		16	14	14	Small coccobacilli
FDM 2-3	0	0	20	26	26	24	28	28	22	Typical
FDM 2-4	2	2	42	96	124	116	112	102	100	Typical
FDM 2-5	0	0	0	0	0	0	0	0	0	
FDM 2-6	2	2	4	2	4	2	2	2	2	
FDM 2-7	0	0	0	0	0	0	0	0	0	
TSB	32	52	60	65	78	75	85	80	90	

FIGURES 3 and 4

Figure 3. Growth of C.bovis strain 14.7 in fully defined medium 2

Open squares, 80 mg of synthetic L-α-lecithin added per litre of FDM 2 (FDM 2-2)
Open circles, 80 mg of synthetic DL-α-lecithin added per litre of FDM 2 (FDM 2-3)
Open triangles, 80 mg of ovolecithin added per litre of FDM 2 (FDM 2-4.)

Broken lines indicate the minimum and maximum growths in tryptose serum broth containing different batches of serum (TSB).

Figure 4. Growth of C.bovis strain 14.7 in fully defined medium 2

Open triangles, 5 ml of Tween 80 added per litre of FDM 2 (FDM 2-8)
Open squares, 0.5 ml of Tween 80 added per litre of FDM 2 (FDM 2-9)
Open circles, 0.05 ml of Tween 80 added per litre of FDM 2 (FDM 2-10)
Plus signs, 0.05 ml of Tween 80 and 80 mg of L-α-lecithin added per litre of FDM 2 (FDM 2-11).

Broken lines indicate the minimum and maximum growths in tryptose serum broth containing different batches of serum (TSB).

FIGURE 3

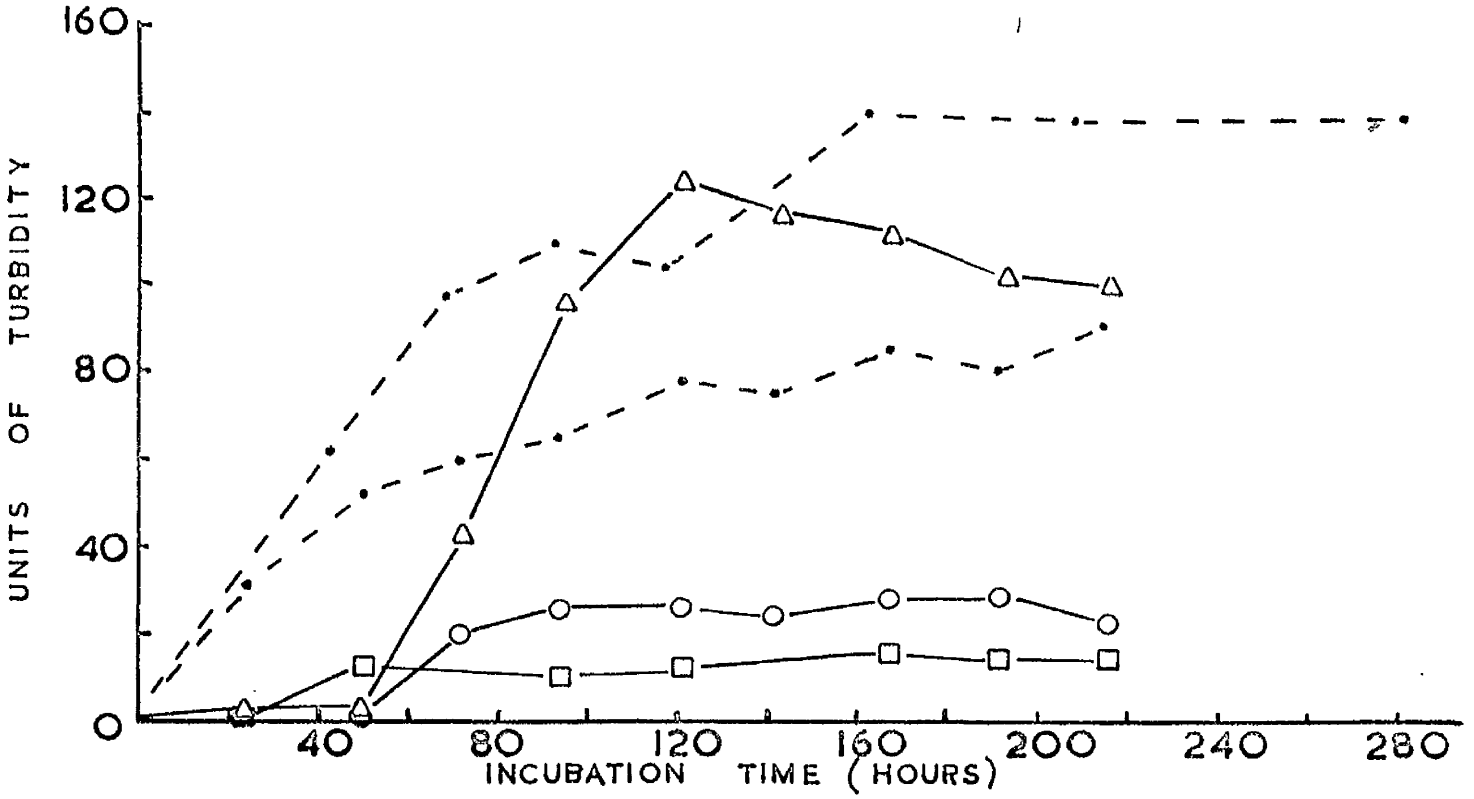
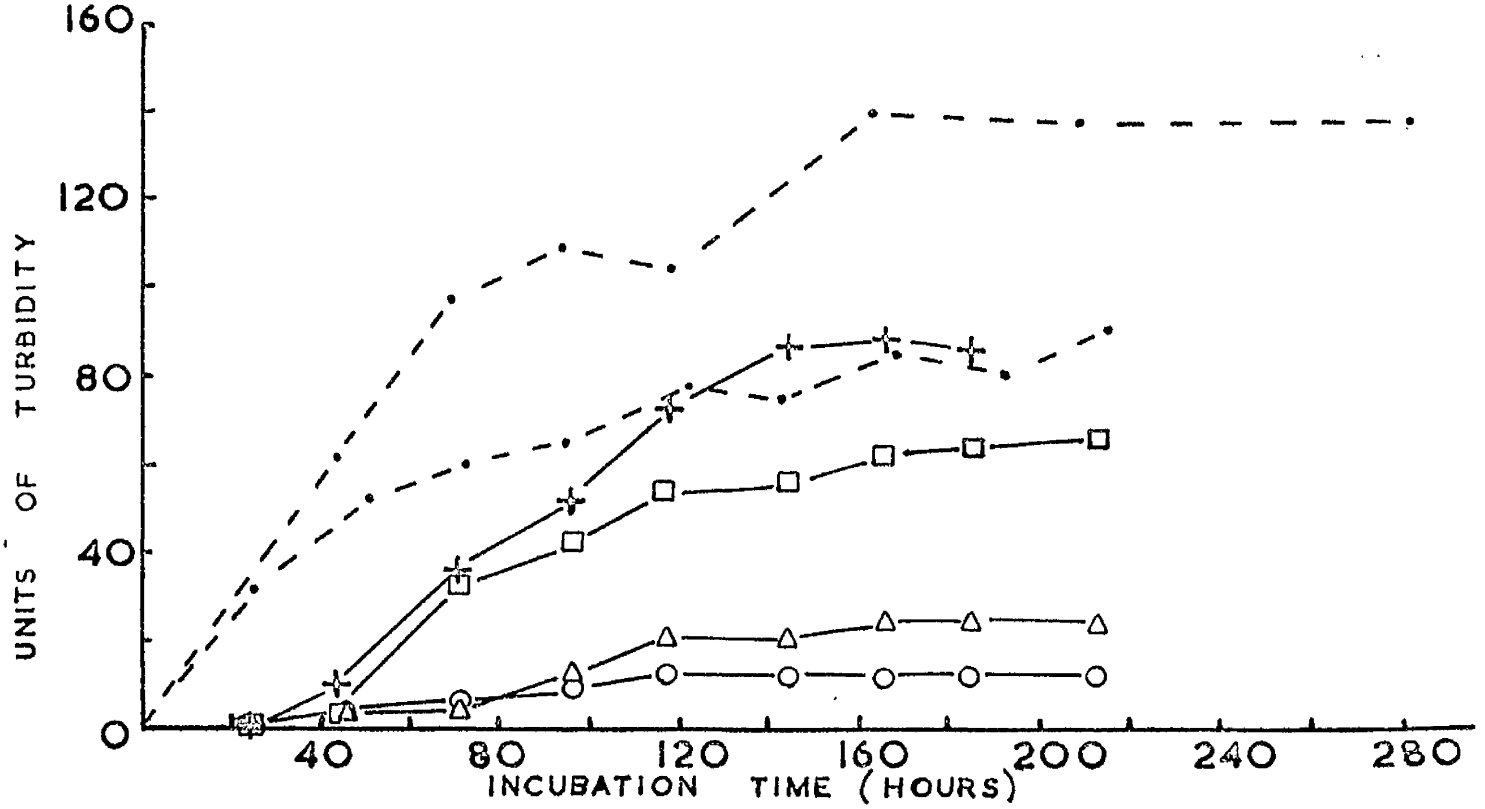


FIGURE 4



to the presence of traces of surface-active substances but mainly to the fatty acids in natural lecithin being heterogeneous, the fatty acids of ovolecithin including oleic acid (43%) and palmitic acid (32%) (Romanoff and Romanoff, 1949). In order to improve the dispersion of the synthetic lecithins in subsequent series media were prepared with the addition of small amounts of Tween 80 as solubilizer followed by the addition of the appropriate synthetic lecithin.

No growth of C.bovis strain 124 occurred in media supplemented with glycerophosphoric acid, sodium β-glycerophosphate or ethanolamine phosphoric acid.

Further series using FDM 2

The medium was supplemented by the following additions:

FDM 2-8 : + Tween 80 (5 ml/l)

FDM 2-9 : + Tween 80 (0.5 ml/l)

FDM 2-10 : + Tween 80 (0.05 ml/l)

FDM 2-11 : + Tween 80 (0.05 ml/l) and L- α-lecithin (80 mg/l)

FDM 2-12 : + Tween 80 (0.05 ml/l) and DL- α-lecithin (80 mg/l)

FDM 2-13 : + Tween 80 (Q05 ml/l) and ovolecithin (80 mg/l)

The growth of C.bovis strain 147 was determined in all six media, and the growth of C.bovis strain 124 was determined in FDM 2-11 and FDM 2-12.

Results

The turbidities of the cultures in Corrected Turbidity Units (CTU) are shown in Table 18, with selected growth curves shown in Figure 4.

Corynebacterium bovis strain 147 grew better in the medium (FDM 2-11)

TABLE 18. GROWTH OF C.BOVIS STRAINS 124 AND 147

IN MEDIUM FDM - 2

Medium and organism	Growth of C.bovis, in CTU, after incubation for the following times (hours)								
	23h	45h	70h	92h	123h	164h	187h	213h	
C.bovis strain 124									
FDM 2-11	0	2	2	0	0	0	0	2	
FDM 2-12	0	2	4	4	-	-	-	-	
	24h	43h	70h	95h	116h	143h	165h	184h	211h
C.bovis strain 147									
FDM 2-8	0	2	4	12	20	20	24	24	24
FDM 2-9	0	2	32	42	54	56	62	64	66
FDM 2-10	0	2	6	8	12	12	12	12	12
FDM 2-11	0	10	36	52	72	86	88	86	-
FDM 2-12	2	0	0	2	2	2	4	18	28
FDM 2-13	0	0	42	74	82	84	84	84	-

in which the L- α-lecithin was solubilized by Tween 80 than it did in the medium (FDM 2-2) which was supplemented by L-α-lecithin alone. The medium containing 0.05% Tween 80 (FDM 2-9) also supported reasonably good growth of C.bovis strain 147. Nevertheless the growth in these two media was only comparable with the lowest growth level obtained with tryptose serum broth. In view of this, and of the negligible growth of C.bovis strain 124 in the lecithin-containing media, the basal medium was modified by the addition of further amino acids.

During this series of experiments, duplicate sets of flasks containing 25 ml amounts and 50 ml amounts of inoculated media showed that the agitation during incubation allowed the amount of medium in a 100 ml Erlenmeyer flask to be increased to 50 ml without noticeably affecting the growth of the bacteria.

Fully defined medium 3 (FDM 3)

The fully defined medium 3 was basically the same as FDM 2 but after sterilization a sterile supplement of 9 amino acids was added aseptically in addition to the Supplements A, B, C and D. The additional amino acids were:

1) α-aminobutyric acid (2-amino-n-butyric acid), an amino acid that has been found repeatedly in animal tissues (Tschiersch & Mothes, 1963) and has also been reported in hydrolysates from Corynebacterium diphtheriae (Work 1949), although its presence in acid hydrolysates could be due to its secondary formation from threonine (Tschiersch & Mothes, 1963);

2) citrulline and ornithine, intermediary products in the urea-cycle;

3) creatinine, potentially useful as an energy source;

4) hydroxyproline, required by a number of bacteria and found by Work (1949) in hydrolysates of C.diphtheriae;

5) Isoleucine, phenylalanine, and valine, regularly found in proteins and required by a number of bacteria; and

7) taurine, found in all proteins.

Amino acid supplement: 0.5 g DL-2-amino-n-butyric acid, 0.5 g DL-citrulline, 0.5 g creatinine, 0.5 g L-hydroxyproline, 0.5 g DL-isoleucine, 0.5 g DL-ornithine hydrochloride, 0.5 g DL-phenylalanine, 0.5 g taurine and 0.5 g DL-valine were dissolved in 100 ml of glass-distilled water and sterilized by filtration. To give a final nominal concentration of 0.05 g of each amino acid per litre of medium, 0.5 ml of the supplement was added to each 50 ml of basal medium.

The growth of C.bovis strains 124 and 147 in this medium was determined without further additions and after supplementation as follows.

FDM 3-1 : No supplementation

FDM 3-2 : + L-α-lecithin (80 mg/l)

FDM 3-3 : + DL-α-lecithin (80 mg/l)

FDM 3-4 : + ovolecithin (80 mg/l)

FDM 3-8 : + Tween 80 (5 ml/l)

FDM 3-9 : + Tween 80 (0.5 ml/l)

FDM 3-10 : + Tween 80 (0.05 ml/l)

FDM 3-11 : + Tween 80 (0.05 ml/l) and L-α-lecithin (80 mg/l)

FDM 3-12 : + Tween 80 (0.05 ml/l) and DL- α -lecithin (80 mg/l)

FDM 3-13 : + Tween 80 (0.05 ml/l) and ovolecithin (80 mg/l)

The effect of glucose. Since there have been many reports of inhibition of bacterial growth being caused by the inclusion of glucose in media before autoclaving (see Meynell and Meynell, 1965) the preparations of the two media FDM 3-9 and FDM 3-11 were also modified by omitting the glucose from the basal medium before autoclaving. The appropriate amount of a filter-sterilized 10% (w/v) solution of glucose was added to the flasks of sterile medium together with the other supplements (FDM 3-9G and FDM 3-11G). Media with the glucose omitted were also inoculated and incubated (FDM 3-9NG and FDM 3-11NG).

Results

The amount of growth obtained in the media, expressed in terms of corrected turbidity units, is shown in Table 19.

The basal medium appeared more satisfactory than FDM 2, with firstly C.bovis strain 124 giving growth in the media containing synthetic lecithin solubilized with Tween 80, and secondly the rate of growth and yield of C.bovis strain 147 in the medium containing 0.05% Tween 80 (FDM 3-9) was much greater than in the equivalent medium using the simpler basal medium (FDM 2-9) and compared well with the amount of growth obtained in serum broth.

Corynebacterium bovis strain 124 was successfully carried through seven successive subcultures in the two media FDM 3-9 and FDM 3-11, and strain 147 was successfully transferred through nine subcultures in these two media. Thus growth of the organisms in the media did not

TABLE 19. GROWTH OF C.BOVIS STRAINS 124 AND 147 IN MEDIUM FDM 3

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)									Microscopic appearance
	23h	45h	70h	92h	123h	164h	187h	213h		
C.bovis strain 124										
FDM 3-1	0	0	0	0	0	0	0	0		
FDM 3-10	0	4	26	34	34	36	34	36		Typical
FDM 3-11	0	0	6	52	74	86	90	82		Typical
FDM 3-12	4	12	68	78	86	84	80	82		Typical
FDM 3-13	4	46	112	114	112	114	114	116		Typical
C.bovis strain 147										
FDM 3-2	2	6	6	6	8	6	6	8		Typical
FDM 3-3	0	6	6	6	4	6	6	6		Typical
FDM 3-4	20	58	80	84	84	82	80	84		Typical
C.bovis strain 124	24h	48h	76h	101h	121h	142h	166h	190h		
FDM 3-2	0	0	4	8	6	4	6	6		
FDM 3-4	0	12	24	32	34	32	34	34		
C.bovis strain 147	23h	43h	70h	95h	116h	143h	165h	184h	211h	
FDM 3-1	0	0	0	0	0	0	0	0	0	
FDM 3-8	2	10	20	20	16	18	16	22	30	Abnormal
FDM 3-9	4	44	106	114	114	112	114	110	-	Typical
FDM 3-10	2	8	14	12	12	12	12	12	-	Abnormal
FDM 3-11	4	24	40	54	78	100	98	96	-	Typical
FDM 3-12	2	30	48	68	88	92	88	90	-	Typical
FDM 3-13	32	62	-	86	84	84	86	88	-	Typical

rely upon a carry-over of essential growth factors with the inoculum or on release of essential growth factors from organisms in the inoculum.

The effect of glucose: There was not a significant difference in the growth rates or yields obtained in the media FDM 3-9 and FDM 3-11 in which the glucose had been added after sterilization of the basal medium, compared with the standard media in which the glucose was included before sterilization. Thus the inhibitory effect discussed by Meynell and Meynell (1965) did not occur with C.bovis strains 124 and 147. No growth occurred when glucose was omitted from the medium.

Determination of vitamin requirements, using modifications of Medium FDM 3

In this series of media the medium used was basically FDM 3-11 (that is, FDM 3 + 0.005% Tween 80 and L-α-lecithin), but the vitamin supplements A, B, C and D were added in various combinations, and also the individual vitamins and growth factors comprising these supplements were added singly and in combination. The media examined were as follows:

1. + Supplements A, B, C and D
2. Without Supplements A, B, C and D
3. + Supplement A
4. + Supplement B
5. + Supplement D
6. + Supplement C
7. + Supplements A, B and D

8. + Supplements A, B and C

9. + Supplements A, C and D

10. + Supplements B, C and D.

Second series (FDM 3-11 with Supplements A, B, C and D omitted)

11. + riboflavin

12. + thiamin

13. + pimelic acid

14. + β-alanine

15. + pyridoxine

16. + riboflavin, thiamin, pimelic acid, β-alanine and pyridoxine

17. + riboflavin, thiamin, pimelic acid and β-alanine

18. + riboflavin, thiamin, pimelic acid and pyridoxine

19. + riboflavin, thiamin β-alanine and pyridoxine

20. + riboflavin, pimelic acid, β-alanine and pyridoxine

21. + thiamin, pimelic acid, β-alanine and pyridoxine

Riboflavin: riboflavin, 2,000 µg, dissolved in 100 ml of 0.02N acetic acid and sterilized by filtration directly into a sterile screw-capped bottle. To obtain a final concentration of 20 µg of riboflavin per litre of medium, 0.05 ml of solution was added to 50 ml of medium.

Thiamin: thiamin hydrochloride, 2,000 µg, dissolved in 100 ml of 0.02 N acetic acid and sterilized by filtration directly into a sterile screw-capped bottle. To obtain a final concentration of 20 µg of thiamin per litre of medium 0.05 ml of solution was added to each 50 ml of medium.

Pimelic acid: pimelic acid, 0.015 g, dissolved in 100 ml of 0.02 N

acetic acid and sterilized by autoclaving at 115°C for 20 minutes. To each 50 ml of medium was added 0.05 ml of solution, to obtain a final concentration of 150 µg of pimelic acid per litre of medium.

β-Alanine: 0.023 g of β-alanine was dissolved in 100 ml of 0.02 N acetic acid and sterilized by autoclaving at 115°C for 20 minutes. To each 50 ml of medium was added 0.05 ml of solution to obtain a final concentration of 230 µg of β-alanine per litre of medium

Pyridoxine (Supplement C): pyridoxine hydrochloride, 0.01 g, dissolved in 100 ml of distilled water and sterilized by filtration directly into a sterile screw-capped bottle. To each 50 ml of medium was added 0.05 ml of solution to give a final concentration of 100 µg of pyridoxine hydrochloride per litre of medium.

Results

The turbidities (expressed as Corrected Turbidity Units) of the cultures in media 1-10 are shown in Table 20a. Corynebacterium bovis strain 124 failed to grow in the absence of supplement A, and thus had a requirement for riboflavin and/or thiamin. No definite conclusions can be drawn concerning the vitamin requirements of C.bovis strain 147, since although no growth was obtained in the absence of the four supplements, the growth patterns of this strain were not consistently correlated with any of the supplements.

The turbidities (expressed as Corrected Turbidity Units) of the cultures in the media 11-21 are shown in Table 20b. The growth of C.bovis strain 124 in media 16, 17, 18 and 19 and absence of significant growth in media 11 and 12 indicates an absolute nutritional requirement

TABLE 20a. GROWTH OF C.BOVIS IN MEDIA CONTAINING VARIOUS

TABLE 20a. GROWTH OF C.BOVIS IN MEDIA CONTAINING VARIOUS

VITAMINS AND GROWTH FACTORS

Medium and organisms	Growth of C.bovis in C.T.U. after incubation for the following time (hours)					
	47h	94h	144h	171h	238h	310h
C.bovis strain 124.						
1	4	40	56	74	88	88
2	0	0	2	2	2	2
3	2	36	56	68	92	92
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	2	34	50	70	72
8	2	32	44	52	60	70
9	6	42	58	72	88	96
10	0	2	2	2	2	2
C.bovis strain 147						
1	2	24	34	36	52	60
2	2	2	2	2	2	2
3	4	18	24	24	32	34
4	0	0	0	0	0	0
5	1	24	30	38	42	62
6	1	18	28	28	40	46
7	1	24	46	56	60	122
8	0	0	0	0	0	0
9	4	28	44	54	76	122
10	1	8	16	18	24	48

TABLE 20b. GROWTH OF C.BOVIS IN MEDIUM CONTAINING VARIOUS VITAMINS AND GROWTH FACTORS

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)						
	48h	72h	143h	144h	191h	241h	335h
C.bovis strain 124							
11	0		0			0	
12	0		4			10	
13	0		0			0	
14	0		0			0	
15	0		0			0	
16	0		38			60	
17	0		38			48	
18	0		10			26	
19	0		40			68	
20	0		0			0	
21	0		0			4	
C.bovis strain 147							
11		6		24	40		72
12		0		2	14		24
13		0		8	20		32
14		0		8	20		24
15		4		16	48		54
16		8		38	60		72
17		6		20	46		64
18		8		30	64		74
19		10		32	60		84
20		6		22	42		48
21		8		28	44		46

by this strain for both riboflavin and thiamin. In the case of C.bovis strain 147, the organism did not have an absolute nutritional requirement for riboflavin or thiamin, but for growth of the organism to be similar to that obtained in FDM 3-11 both riboflavin and thiamin were required.

Fully defined medium 4 (FDM 4) and the effect of biotin and m-inositol

A fully defined medium was formulated which included all the amino acids which have been shown to be essential for the growth of certain bacteria (Hugo, 1964) and recommended by Meynell and Meynell (1965) for inclusion in chemically defined media. α-Aminobutyric acid, creatinine, citrulline, ornithine and taurine were excluded from this medium. The medium also included xanthine.

The medium had the following constitution:

Sodium chloride (AR grade)	5.0 g	Glycine (AR grade)	0.05 g
Potassium dihydrogen phosphate (AR grade)	0.25 g	L-Histidine HCl	0.05 g
Dipotassium hydrogen phosphate	0.35 g	L-Hydroxyproline	0.05 g
Ammonium dihydrogen phosphate (AR grade)	0.5 g	DL-Isoleucine	0.05 g
Magnesium sulphate, hydrated ($MgSO_4.7H_2O$)(AR grade)	0.25 g	DL-Leucine	0.05 g
Copper sulphate, hydrated ($CuSO_4.5H_2O$)(AR grade)	0.003g	L-Lysine HCl	0.05 g
		DL-Methionine	0.05 g
Ferrous sulphate, hydrated ($FeSO_4.7H_2O$)(AR grade)	0.007g	DL-β-Phenylalanine	0.05 g
		L-Proline	0.05 g
D-Glucose (AR grade)	1.0 g	DL-Serine	0.05 g
DL-α-Alanine	0.05 g	DL-Threonine	0.05 g
L-Arginine HCl	0.05 g	DL-Tryptophan	0.05 g
L-Asparagine	0.05 g	Tyrosine	0.05 g
Aspartic acid	0.05 g	Valine	0.05 g
L-Cystine	0.05 g	Adenine	0.01 g
L-Cysteine HCl	0.05 g	Choline chloride	0.01 g

L-Glutamic acid	0.05 g	Guanine	0.01 g
Glutamine	0.05 g	Xanthine	0.01 g
Distilled water	to 1 litre	Uracil	0.01 g

As in the case of PDM 1, the ingredients (with the exception of cystine, guanine and xanthine) were dissolved one at a time in 750 ml of glass-distilled water at room temperature which was kept continuously stirred using a magnetic stirrer. The cystine, guanine and xanthine were added to the minimum amount of 20% (w/v) sodium hydroxide (AR grade reagent dissolved in glass-distilled water) required to obtain complete solution, which was then mixed with the water containing all the other ingredients. The volume was made up to 1 litre with glass-distilled water. The medium was autoclaved at 107°C for 5 minutes. After the medium had cooled, the reaction of the medium was adjusted to pH 7.2 using N sodium hydroxide (AR grade) and N hydrochloric acid (AR grade).

The medium was then clarified through an asbestos filter (Carlson-Ford filter grade K5) and distributed in 50 ml amounts in 100 ml Erlenmeyer flasks fitted with stainless steel closures. The flasks of medium were sterilized by autoclaving at 115°C for 20 minutes. The four Supplements A, B, C and D were added to the medium. All four vitamin supplements were added since although the vitamin requirements of C.bovis strain 124 had been determined, the exact requirements of C.bovis strain 147 remained obscure. To each 50 ml of medium were added 0.05 ml of Supplement A (giving nominal final concentrations of riboflavin 20 µg/l; thiamin hydrochloride 20 µg/l); 0.5 ml of Supplement B (giving nominal final concentrations of calcium

pantothenate 200 µg/l; nicotinic acid 2 mg/l; folic acid, 20 µg/l pyridoxal hydrochloride 600 µg/l; ρ-aminobenzoic acid, 200 µg/l); 0.05 ml of Supplement C (giving a nominal final concentration of 100 µg/l); 0.05 ml of Supplement D (giving nominal final concentrations of pimelic acid 150 µg/l; β-alanine, 230 µg/l).

At this stage in the experiments, the basal medium was sufficiently good to enable the fatty acid-sparing action of biotin to be examined. It has been known for some time that biotin is concerned in fatty acid synthesis, and that many bacteria have a requirement for oleic acid or biotin (Williams et al., 1947; Snell 1951). Pimelic acid is a precursor for biotin synthesis and C.diphtheriae has a requirement for biotin or pimelic acid, since it can synthesize biotin from pimelic acid (Mueller, 1940). In the present investigation it seemed unlikely that biotin could replace the apparent fatty acid requirement, since firstly no growth was obtained in a broth containing Panmede, a liver digest containing biotin, secondly no significant growth was obtained in the partially defined medium to which yeast extract had been added, and finally pimelic acid was always included in the partially and fully defined media. In order to further examine the effect of biotin, the media FDM 3 and FDM 4 were supplemented as indicated below.

FDM 3-1 and FDM 4-1 : no supplementation

FDM 3-2 and FDM 4-2 : + L-α-lecithin (80 mg/l)

FDM 3-2B and FDM 4-2B : + L-α-lecithin (80 mg/l) and biotin (35 µg/l)

FDM 3-9 and FDM 4-9 : + Tween 80 (0.5 ml/l)

FDM 3-9B and FDM 4-9B : + Tween 80 (0.5 ml/l) and biotin (35 µg/l)

FDM 3-14 and FDM 4-14 : + biotin (35 µg/l)

FDM 3-15 and FDM 4-15 : + m-inositol (60 mg/l)

FDM 3-16 and FDM 4-16 : + biotin (35 µg/l) and m-inositol (60 mg/l)

The growth of C.bovis strains 124 and 147 was examined in the above media.

Biotin: biotin (Sigma Chemical Co., 12, Lettice St., London, S.W.6), 3.5 mg, dissolved in 100 ml of glass-distilled water and sterilized by filtration. To each 50 ml of medium, 0.05 ml of solution was added to give a nominal final concentration of 35 µg biotin per litre of medium.

m-Inositol: m-inositol, 600 mg, dissolved in 100 ml of glass-distilled water and sterilized by filtration. To obtain a nominal final concentration of 60 mg m-inositol per litre of medium, 0.5 ml of solution was added to each 50 ml of medium.

Results

The turbidities of the cultures, expressed in Corrected Turbidity Units are shown in Table 21 with selected growth curves shown in Figure 5. No growth occurred in FDM 3-1, FDM 4-1, FDM 3-2, FDM 4-2, FDM 3-2B, FDM 4-2B, FDM 3-14, FDM 4-14, FDM 3-15, FDM 4-15, FDM 3-16 and FDM 4-16. Thus biotin is incapable of replacing the requirement of these organisms for Tween or other fatty acid-containing compounds, although biotin is known to be involved in fatty acid metabolism (Snell, 1951; Beerstecher, 1962) and as already mentioned biotin has been shown to replace the oleic acid requirement of a number of

TABLE 21. GROWTH OF C.BOVIS IN MEDIA FDM 3 AND FDM 4.

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)						
	44h	70h	95h	122h	141h	188h	212h
C.bovis strain 124							
FDM 3-9	11	28	44	62	80	-	85
FDM 4-9	16	35	48	59	64	88	88
FDM 3-9B	20	48	61	92	101	128	-
FDM 4-9B	32	60	87	128	145	154	-
C.bovis strain 147							
FDM 3-9	0	0	4	15	20	32	-
FDM 4-9	4	20	31	42	67	83	89
FDM 3-9B	0	2	49	71	89	108	142
FDM 4-9B	0	15	72	113	142	172	162
TSB	23	69	81	88	93	99	97

FIGURE 5

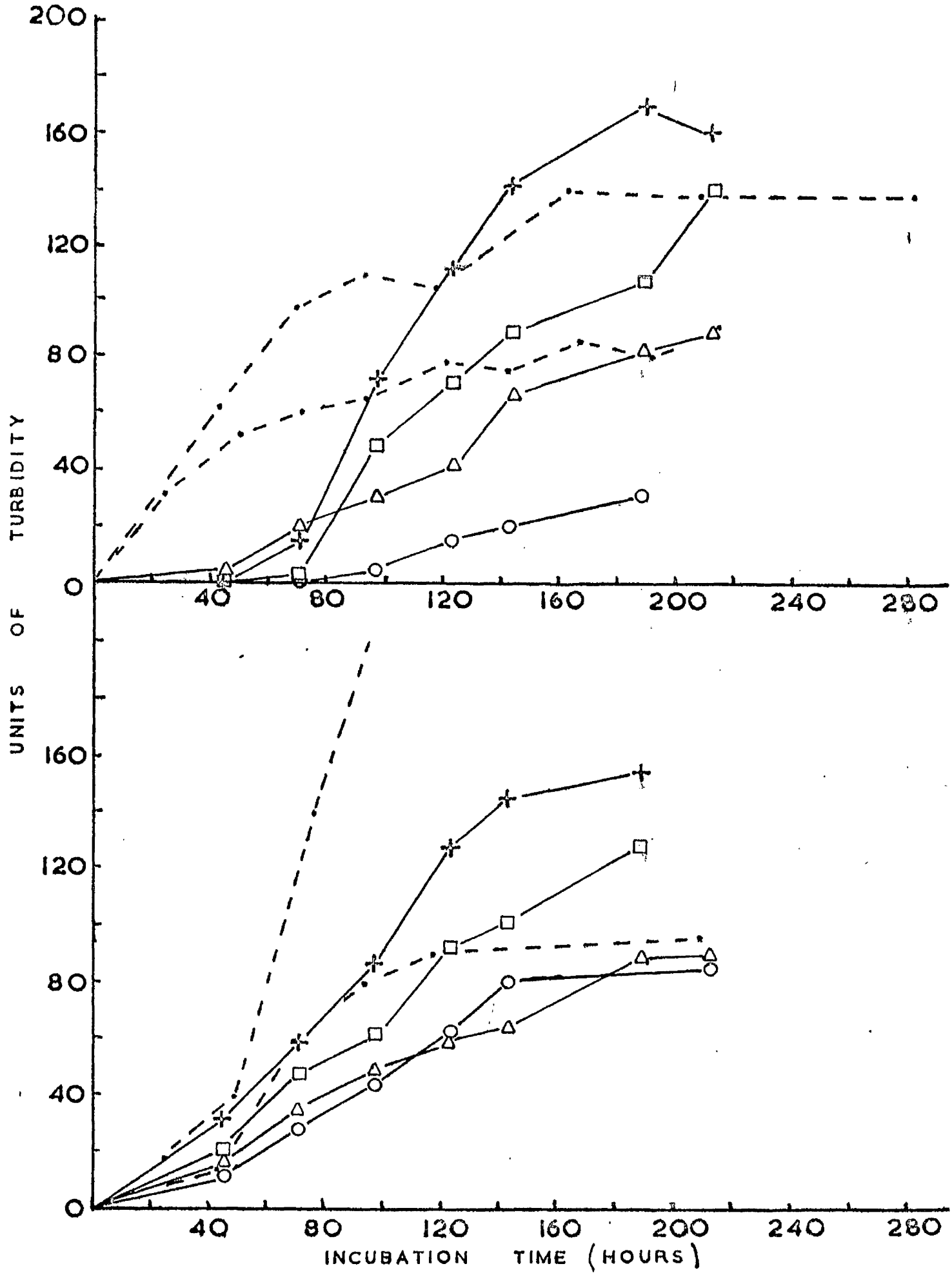
Figure 5. Growth of C.bovis strains 147 and 124 in fully defined media 3 and 4

a) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 147 in FDM 3 and FDM 4.

b) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 124 in FDM 3 and FDM 4..

Open circles,	0.5 ml of Tween 80 added per litre of FDM 3 (FDM 3-9)
Open triangles,	0.5 ml of Tween 80 added per litre of FDM 4. (FDM 4-9)
Open squares,	0.5 ml of Tween 80 and 35 µg of biotin added per litre of FDM 3 (FDM 3-9B)
Plus signs,	0.5 ml of Tween 80 and 35 µg of biotin added per litre of FDM 4. (FDM 4-9B).

FIGURE 5


200
160
120
80
40
0
UNITS OF TURBIDITY
40
80
120
160
200
240
280
160
120
80
40
0
40
80
120
160
200
240
280
INCUBATION TIME (HOURS)

organisms (Williams et al., 1947). Thus these strains of C.bovis are similar to the "minute" streptococci and Lactobacillus leichmannii which were found by Deibel and Niven (1955) to require an unsaturated fatty acid (provided, for example, as Tween 80) even in the presence of biotin.

Although the organisms do not have an absolute requirement for biotin, the inclusion of biotin in media containing Tween 80 resulted in much higher maximum turbidities, and higher growth rates (Table 21).

Effect of varying the phosphate concentrations in, and adding citrate to, FDM 5

The fully defined medium FDM 5 was basically the same as FDM 4, but since the addition of biotin was shown in the previous experiment to promote better growth of both strains, biotin (35 µg/l) was added to FDM 4 in addition to the four Supplements A, B, C and D. This medium was termed FDM 5.

The effect of varying the concentrations of sodium chloride, dipotassium hydrogen phosphate and potassium dihydrogen phosphate in, and the effect of adding citrate to, the fully defined medium FDM 5 was examined. The modified concentrations of phosphates and the concentration of citrate were similar to those employed by Davis and Mingioli (1950). The original and modified basal media contained these compounds in the following amounts (per litre of medium).

Compound	Medium				
	FDM 5	FDM 5a	FDM 5b	FDM 5c	FDM 5d
Sodium chloride	5.0	5.0	5.0	5.0	3.0
Potassium dihydrogen phosphate	0.25	0.25	3.0	3.0	3.0
Dipotassium hydrogen phosphate	0.35	0.35	7.0	7.0	7.0
Sodium citrate, hydrated		0.5		0.5	0.5
pH:	7.20	7.05	7.05	7.05	7.10

The two strains of C.bovis, strains 124 and 147, were grown in this range of media after the addition of Tween 80 (0.5 ml/l)(FDM 5-9 etc.), or Tween 80 (0.05 ml/l) and synthetic L-α-lecithin (80 mg/l)(FDM 5-11 etc). The effect of omitting glucose from these media was also examined to confirm the glucose requirement of C.bovis noted on page 101.

Results:

The turbidities of the cultures, expressed in Corrected Turbidity Units, are shown in Table 22. No growth occurred in any of the media without further supplementation.

None of the modifications resulted in a significant improvement in the growth rates or total yields of both strains, and the higher concentration of phosphates resulted in lower yields particularly in the medium containing 0.05% Tween 80.

The stimulative effect of biotin noted on page 108 was confirmed in this series of cultures.

No growth occurred in any of the media when glucose was omitted, thus confirming the requirement of these organisms for glucose.

This basal medium, FDM 5, appeared to be satisfactory and with the addition of Tween 80 (FDM 5-9) or synthetic L-α-lecithin and Tween 80 (FDM 5-11) good growth of C.bovis was obtained. It was therefore used to further investigate the fatty acid requirement of the organisms (page 112).

The growth of another isolate of C.bovis (strain 159), the type culture C.bovis NCTC 3224 and the diphtheroid isolated from human milk

TABLE 22. GROWTH OF C.BOVIS IN FDM 5, AND MODIFIED FDM 5

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)				
	49h	73 h	123h	172h	241h
C.bovis strain 124					
FDM 5-9	52	83	108	150	158
FDM 5a-9	70	90	146	166	166
FDM 5b-9	38	62	110	122	132
FDM 5c-9	30	55	106	106	106
FDM 5d-9	6	38	128	140	150
FDM 5a-11	45	68	94	93	103
FDM 5b-11	62	68	88	100	104
FDM 5c-11	50	58	72	82	94
FDM 5d-11	10	40	79	84	104
TSB	36	62	78	90	-
C.bovis strain 147					
FDM 5-9	48	106	192	226	250
FDM 5a-9	48	102	220	238	240
FDM 5b-9	66	94	146	165	170
FDM 5c-9	56	82	142	154	150
FDM 5d-9	5	36	121	158	158
FDM 5-11	50	75	116	125	133
FDM 5a-11	39	68	111	123	133
FDM 5b-11	54	71	120	122	126
FDM 5c-11	43	75	111	115	119
FDM 5d-11	8	40	96	104	116
TSB	108	110	110	120	118

was also examined in the lecithin- and Tween-containing media.

The growth of other diphtheroids in fully defined media

The isolates of C.bovis strains 124, 147 and 159, the diphtheroid isolated from human milk (strain 173), and C.bovis NCTC 3224 were grown in FDM 5-1 (no supplementation), FDM 5-9 (+ 0.05% v/v Tween 80), FDM 5-11 (+ 0.008% w/v synthetic L-α-lecithin, and 0.005% v/v Tween 80), and tryptose serum broth.

Results

The amounts of growth obtained expressed in Corrected Turbidity Units are shown in Table 23. No growth of the human diphtheroid strain 173 occurred in the fully defined media and the maximum turbidity of this strain in tryptose serum broth was only 6 C.T.U.

Tween 80 and lecithin as surfactants

Since the substances shown to satisfy an essential growth requirement of C.bovis, namely serum, lecithin and Tween 80, all have surfactant properties in addition to containing fatty acid residues, the effect was examined of culturing C.bovis, strains 124 and 147, in fully defined media containing various non-ionic surface-active agents, with HLB (hydrophile-lipophile balance) values similar to that of Tween 80 (HLB 15). The basal medium used was FDM 5, and this was supplemented by the addition of the surface-active agents listed below, each in concentrations of 0.05 g per litre of medium and 0.5 g per litre of medium.

Brij 35 (Atlas Chemical Industries Inc., Wilmington, Delaware), a polyoxyethylene (23) lauryl ether, HLB 16.9;

TABLE 23 GROWTH OF CORYNEBACTERIA IN LECITHIN - AND TWEEN - CONTAINING FULLY DEFINED MEDIA

Organism and period of incubation (in hours)	Growth, in C.T.U., after incubation in the media indicated.			
	FDM 5-1	FDM 5-9	FDM 5-11	TSB
C.bovis strain 124				
47 hours	0	54	36	29
119 hours	0	160	108	89
Maximum turbidity obtained	0	184	160	147
C.bovis strain 147				
47 hours	0	60	38	57
119 hours	0	160	112	115
Maximum turbidity obtained	0	252	200	145
C.bovis strain 159				
47 hours	0	32	28	5
119 hours	0	202	96	39
Maximum turbidity obtained	0	250	216	123
C.bovis NCTC 3224				
47 hours	0	34	42	149
119 hours	0	74	126	243
Maximum turbidity obtained	0	118	192	313

Brij 58 (Atlas), a polyoxyethylene (20) cetyl ether, HLB 15.7;

Brij 78 (Atlas), a polyoxytheylene (20) stearyl ether, HLB 15.3;

Brij 98 (Atlas), a polyoxyethylene (20) oleyl ether, HLB 15.3;

Texofor A16, Texofor A24 and Texofor 65A30P (Glovers Chemicals Ltd., Wortley Low Mills, Leeds 12), polyoxyethylene cetyl ethers;

Texofor B16 and Texofor B23 (Glovers), polyoxyethylene lauryl ethers;

Texofor D60 (Glovers), a polyoxyethylene ether of a glyceride oil;

Texofor E23 (Glovers), a polyoxyethylene ether of a saturated fatty acid;

Texofor FN15, Texofor FX170 and Texofor FX300 (Glovers), polyoxyethylene ethers of alkyl phenols;

Texofor N8 and Texofor N12 (Glovers), polyoxyethylene ethers of fatty alcohols;

Triton X100 (Rohm and Haas Co., Philadelphia), a polyoxyethylene (10) octyl phenyl ether.

The surface-active agents which were liquid at room temperature were sterilized in 1-oz screw-capped bottles by autoclaving at 110°C for 15 minutes. Those surface-active agents which were solid at room temperature were similarly sterilized but as aqueous 10% (w/v) solutions.

Results

Growth was obtained in only a single medium - that containing Texofor D60. The turbidities obtained (expressed in C.T.U.) are shown in Table 24.

Since the lipophilic base of Texofor D60 consists of a glyceride

TABLE 24. GROWTH OF C.BOVIS IN THE PRESENCE OF TEXOFOR D.60

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)						
	44h	70h	95h	122h	141h	188h	212h
C.bovis strain 124							
FDM 5 + 0.005% Texofor D60	2	8	8	12	12	11	-
FDM 5 + 0.05% Texofor D60	8	22	32	52	49	45	44
TSB	17	47	91	95	119	125	-
C.bovis strain 147							
FDM 5 + 0.005% Texofor D60	0	2	3	4	5	4	-
FDM 5 + 0.05% Texofor D60	2	3	8	8	7	7	-
TSB	23	69	81	88	93	99	97

oil, these results would appear to confirm that the requirement of C.bovis is for fatty acid, although it is interesting to note that no growth of the two strains was obtained in the presence of Brij 98 (0.005% and 0.05%), in which oleyl alcohol was the basis for the lipophilic moiety.

The fatty acid requirement of C.bovis

In order to confirm the requirement of C.bovis for oleic acid or other fatty acid, the growth of C.bovis strains 124 and 147, and C.bovis NCTC 3224 was determined in the fully defined medium FDM 5 to which oleic acid or sodium oleate had been added in various concentrations with and without detoxifying agents. The concentration of a fatty acid capable of stimulating growth of an organism requiring that compound is frequently only slightly less than the concentration causing inhibition of growth, both concentrations usually being very low. Consequently many workers have found it necessary to include in their media substances specifically for the purpose of removing the toxic effects of the fatty acids. For example Hutner (1942) employed saponin and sodium taurocholate as detoxifying agents, Davis and Dubos (1947) used albumin, and Tween 40 was used by Williams and his colleagues (1947). If a detoxifying agent is used, a medium may include a concentration of fatty acid greater than that possible in the absence of the detoxifier and still support growth. Williams and co-workers (Williams and Fieger, 1946; Williams et al., 1947) in a study of the growth of Lactobacillus could obtain growth of the organisms in the presence of 10 µg of oleic acid per ml of medium when a detoxifying agent was included, but the

concentration of oleic acid had to be reduced to 1 µg per ml of medium in the absence of a detoxifier. Meynell and Meynell (1965) have suggested the inclusion of fatty acids at a concentration of 10^{-6}M, or 10^{-4}M when an adsorbent or detoxifier was used.

The media examined were the following:

FDM 5-17a : + oleic acid (40 mg/l)

FDM 5-17b : + oleic acid (10 mg/l)

FDM 5-17c : + oleic acid (0.5 mg/l)

FDM 5-17d : + oleic acid (0.25 mg/ l)

FDM 5-18 : + sodium oleate (80 mg/l)

FDM 5-19 : + oleic acid (40 mg/l) and albumin (1.0 g/l)

FDM 5-19a : + oleic acid (10 mg/l) and albumin (1.0 g/l)

FDM 5-19b : + oleic acid (0.5 mg/l) and albumin (1.0 g/l)

FDM 5-20 : + sodium oleate (80 mg/l) and albumin (1.0 g/l)

FDM 5-21a : + oleic acid (10 mg/l) and sodium taurocholate (1.0 g/l)

FDM 5-21b : + oleic acid (0.5 mg/l) and sodium taurocholate (1.0 g/l)

FDM 5-22a : + oleic acid (10 mg/l) and saponin (1.5 g/l)

FDM 5-22b : + oleic acid (0.5 mg/l) and saponin (1.5 g/l)

FDM 5-23a : + oleic acid (10 mg/l) and Tween 40 (1.0 ml/l)

FDM 5-23b : + oleic acid (0.5 mg/l) and Tween 40 (1.0 ml/l)

FDM 5-23c : + Tween 40 (1.0 ml/l) only

FDM 5-24 : + Tween 20 (0.5 ml/l)

FDM 5-9 : + Tween 80 (0.5 ml/l)

FDM 5-9a : + Tween 80 (0.5 ml/l) and albumin (1.0 g/l)

FDM 5-9b : + Tween 80 (0.5 ml/l) and sodium taurocholate (1.0 g/l)

FDM 5-9c : + Tween 80 (0.5 ml/l) and saponin (1.5 g/l)

Oleic acid: oleic acid, 96% pure from olive oil (Sigma Chemical Co., 12 Lettice St., London, S.W.6), dissolved in 95% ethanol (AR grade) and sterilized by filtration. For a final concentration of 10 mg oleic acid per litre of medium, 0.05 ml of a 1% ethanolic solution was added to each 50 ml of medium; for a final concentration of 0.5 mg oleic acid per litre of medium, 0.05 ml of a 0.05% ethanolic solution was added to each 50 ml of medium.

Albumin: bovine albumin, fraction V (Armour Pharmaceutical Co. Ltd., Eastbourne), a 10% w/v solution in glass-distilled water, sterilized by filtration. To each 50 ml of medium, 0.5 ml of solution was added to give a final concentration of 1.0 g albumin per litre.

Sodium taurocholate: a 10% (w/v) aqueous solution of sodium taurocholate (British Drug Houses Ltd.), sterilized by filtration; 0.5 ml was added to 50 ml of medium for a concentration of 1.0 g of sodium taurocholate per litre.

Saponin: a 15% (w/v) aqueous solution of saponin (British Drug Houses Ltd.), sterilized by filtration; 0.5 ml was added to 50 ml of medium for a nominal final concentration of 1.5 g of saponin per litre of medium.

Sodium oleate: prepared from oleic acid, 96% pure, (Sigma) as described on page 87, but sterilized by filtration.

Tween 20, Tween 40, and Tween 80: polyoxyethylene sorbitan monolaurate (Tween 20), polyoxyethylene sorbitan monopalmitate (Tween 40), or polyoxyethylene sorbitan mono-oleate (Tween 80)(Atlas) sterilized by

autoclaving at 121°C for 15 minutes.

Results

No growth was obtained in the media (FDM 5-17) containing oleic acid at any of the concentrations used, without the addition of a detoxifying agent. When a detoxifying agent was added growth was obtained, the turbidities of the cultures, expressed in Corrected Turbidity Units (C.T.U.) being shown in Table 25 with selected growth curves shown in Figure 6. No growth of the organisms occurred in FDM 5-23c, in which Tween 40 was incorporated alone, demonstrating that the better growth obtained in FDM 5-23a was possibly due to the better detoxifying powers of Tween 40.

The turbidities of the cultures in the Tween 80-containing medium FDM 5-9 in which the fatty acid-detoxifying compounds had been included are shown in Table 26, which also includes the turbidities of the cultures in the medium containing Tween 20.

No growth occurred in media containing the lower concentration of oleic acid (0.5 mg/l) when albumin or Tween 40 was used as the detoxifying agent, although slight growth occurred when saponin was used. Since however, the saponin was a compound obtained from natural sources and was of unspecified purity, growth could have occurred as a result of the presence of growth-stimulating substances present in the saponin preparation as impurities.

Sodium oleate did not support or allow growth in the concentration used (80 mg/l), even when albumin was added, but oleic acid at a concentration of 40 mg/l was inhibitory in the presence of albumin.

TABLE 25. GROWTH OF C.BOVIS IN MEDIA CONTAINING OLEIC ACID

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)						Microscopic appearance
	28h	53h	95h	122h	167h	230h	
C.bovis strain 124							
FDM 5-19a	0	12	20	20	20	20	Gram-positive coccobacilli
FDM 5-19b	0	0	0	0	0	0	
FDM 5-22a	0	0	3	19	24	41	
FDM 5-22b	0	0	0	3	3	7	
FDM 5-23a	8	56	70	68	68	80	Typical
FDM 5-23b	0	0	0	0	0	0	
TSB	26	68	82	80	82	88	
C.bovis strain 147							
FDM 5-19a	12	39	48	56	64	60	Gram-positive coccobacilli
FDM 5-19b	0	0	0	0	0	0	
FDM 5-22a	0	0	28	42	53	52	
FDM 5-22b	0	0	10	10	12	10	
FDM 5-23a	25	66	92	100	96	96	Rods, weakly Gram-positive
FDM 5-23b	0	0	0	0	0	0	
TSB	55	86	100	112	-	128	
C.bovis NCTC 3224							
FDM 5-19a	20	58	64	68	70	70	Gram-positive coccobacilli
FDM 5-19b	0	0	0	0	0	0	
FDM 5-22a	0	0	3	34	40	50	
FDM 5-22b	0	0	8	12	12	13	
FDM 5-23a	4	58	82	102	120	146	Typical
FDM 5-23b	0	0	0	0	0	0	
TSB	49	146	188	212	212	222	

FIGURE 6

Figure 6. Growth of C.bovis strains 147, 124 and NCTC 3224 in fully defined medium 5

a) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 147 in FDM 5.

b) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis strain 124 in FDM 5.

c) The growth determined turbidimetrically (expressed in Corrected Turbidity Units) of C.bovis NCTC 3224 in FDM 5.

Open squares, 10 mg of oleic acid and 10 g of albumin added per litre of FDM 5 (FDM 5-19a)
Open circles, 10 mg of oleic acid and 1.5 g of saponin added per litre of FDM 5 (FDM 5-22a)
Open triangles, 10 mg of oleic acid and 1.0 ml of Tween 40 added per litre of FDM 5 (FDM 5-23a).

FIGURE 6

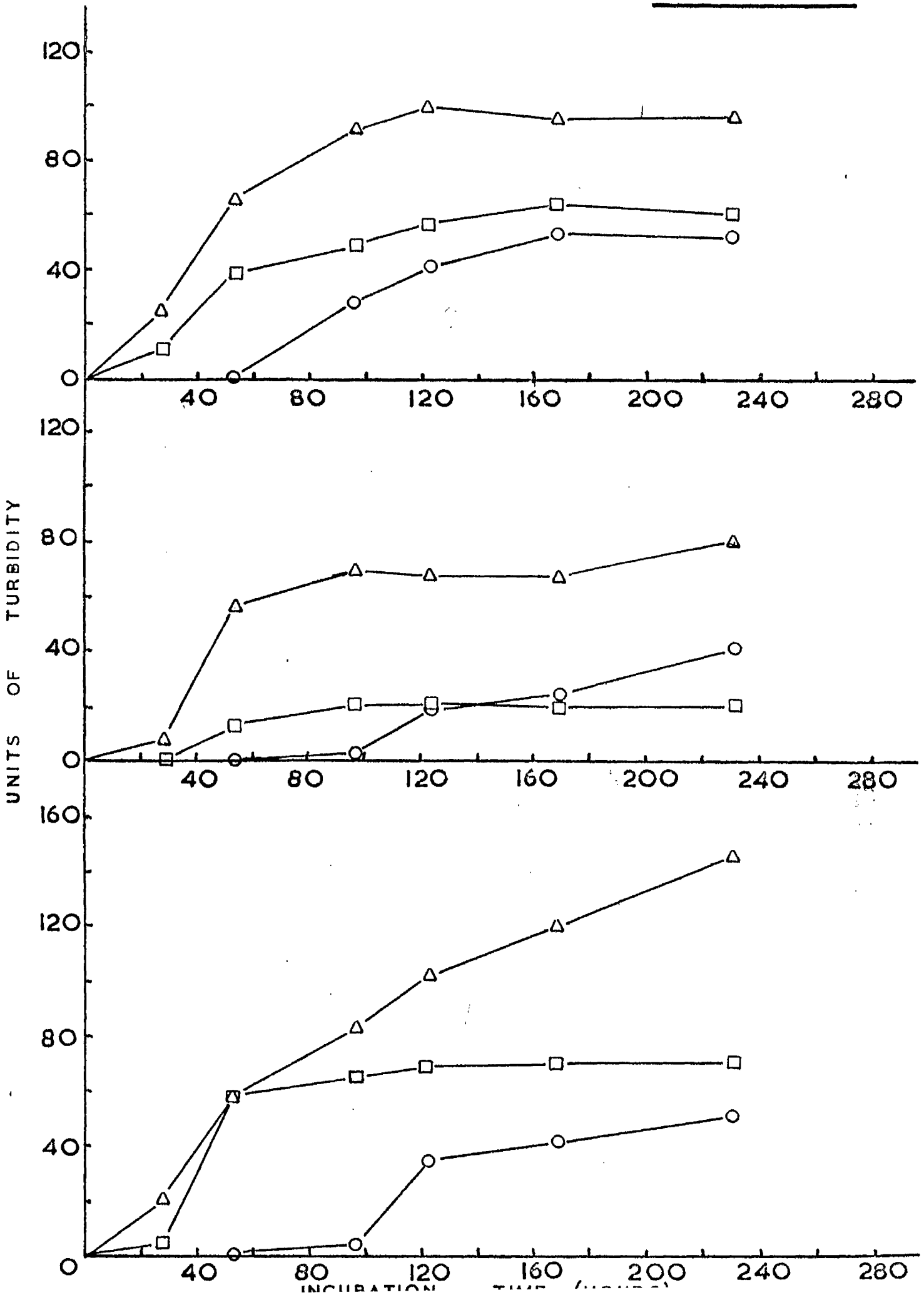

UNITS OF TURBIDITY
120
80
40
0
40
80
120
160
200
240
280
120
80
40
0
40
80
120
160
200
240
280
160
120
80
40
0
40
80
120
160
200
240
280

TABLE 26. GROWTH OF C.BOVIS IN FDM 5-9 WITH FATTY ACID DETOXIFIERS AND IN FDM 5-24

Medium and organism	Growth of C.bovis in C.T.U. after incubation for the following times (hours)						Microscopic appearance
	28h	53h	95h	122h	167h	230h	
C.bovis strain 124							
FDM 5-9	5	48	75	105	145	150	Typical
FDM 5-9a	0	5	22	40	68	74	Gram-positive coccobacilli
FDM 5-9b	15	73	145	165	177	177	Typical
FDM 5-9c	0	0	114	147	171	175	Gram-variable coccobacilli
FDM 5-24	0	0	1	11	51	59	
C.bovis strain 147							
FDM 5-9	20	118	230	266	266	-	Typical
FDM 5-9a	0	12	68	92	136	164	Gram-positive coccobacilli
FDM 5-9b	28	160	352	352	356	384	Gram-positive coccobacilli
FDM 5-9c	0	0	100	142	223	308	Weakly Gram-positive, coccobacilli
FDM 5-24	0	0	4	32	64	78	
C.bovis NCTC 3224							
FDM 5-9	8	54	122	136	136	134	Typical
FDM 5-9a	0	12	48	60	72	76	Typical
FDM 5-9b	28	84	178	180	188	200	Typical
FDM 5-9c	0	0	32	52	88	116	Weakly Gram-positive, coccobacilli
FDM 5-24	0	0	4	6	8	8	

The three detoxifying agents tested in the presence of Tween 80 (see Table 26) all had an effect upon the morphology of at least one of the strains. Of the two detoxifying agents capable of allowing growth in the presence of oleic acid, Tween 40 did not result in morphological variation, whereas when albumin was used the cultures consisted largely of coccobacillary forms and the typical longer club-shaped rods were not seen.

Discussion and conclusions

The study of the nutritional requirement of C.bovis by the development of chemically defined media appears not to have been attempted previously. Although Stokstad, Hoffmann and Belt (1950) have investigated the nutritional requirements of an organism which was tentatively identified by them as being "closely related to Corynebacterium bovis", this diphtheroid did not require supplementation of yeast extract glucose agar with serum or other fatty acid-containing substances to be able to grow. This fact alone suggests that their organism was not identical with C.bovis since the cultural requirement of C.bovis for supplementation of nutrient agar with serum, egg-yolk, Tween, butter-fat or similar substance would appear to be a consistent and constant characteristic of strains of C.bovis. In addition, Jayne-Williams and Skerman (1966) have found that Stokstad's isolate (deposited in the American Type Culture Collection as ATCC 13722) is different from C.bovis in a number of biochemical characteristics.

As pointed out in Section C the enhancement of growth of C.bovis by the addition of serum to nutrient media was observed long ago by Evans (1916) and Bendixen (1933). During the examination of the cultural and biochemical characteristics of C.bovis isolates in the present work it was found that serum could be replaced by butter-fat, egg-yolk, or Tween 80 (or Tween 20) as additives to media such as nutrient agar or tryptose lemco agar which allowed the growth of the isolates. Furthermore these substances were not acting by detoxifying fatty acids present as traces in the nutrient media

(Pollock, 1949) since the addition of activated charcoal (which would adsorb any free fatty acids present) to tryptose lemco agar or of albumin to a nutrient broth (Pannede broth) did not allow the growth of the isolates. Thus it appeared probable that this represented a true nutritional requirement of the isolates, probably for a fatty acid.

Since the chemically defined medium capable of supporting the growth of C.bovis was likely to be complex, and perhaps would be diphasic if lecithin or fatty acids were included, it was considered impracticable to employ solid defined media for determining the nutritional requirements of the organisms. The use of liquid defined media on the other hand, allows the possibility of the development of turbidity as the result of the growth and multiplication of non-exacting mutants. Had very small inocula been employed, the longer lag phase and the extension of the time before the turbidities became readable would have increased the possibility of the development of such non-exacting nutrients and their emergence as the predominant population during the period of incubation. Consequently, and also for the other reason given on page 79, comparatively large inocula were used. Nevertheless, results in which the culture became turbid after a prolonged lag phase must be interpreted with care.

Although the amino acid requirements of the C.bovis isolates were not determined accurately, the development of the basal media has enabled certain conclusions to be drawn. For example, no growth occurred in the first fully defined medium, but growth occurred when histidine, methionine, serine, threonine and tryptophan were included.

These results differed from those obtained by Skerman and Jayne-Williams (1966) who found that all the strains they tested, although stimulated by the inclusion of amino acids, were able to utilize ammonia as a sole nitrogen source when this was present as ammonium sulphate in a concentration of 1 g per litre of medium. In this respect it should be noted that FDM 1 contained 0.5 g of ammonium dihydrogen phosphate per litre of medium in addition to eleven amino acids. Better growth still occurred in the media based on FDM 3, which contained 25 amino acids. However the best growth occurred in FDM 4 and FDM 5 which contained, in addition to the amino acids in FDM 2, glutamine, hydroxyproline, isoleucine, phenylalanine, tyrosine and valine. The improvement of growth obtained in FDM 4, compared with FDM 3, could have been due to the omission of one or more of the amino acids α-aminobutyric acid, citrulline, creatinine, ornithine or taurine, but it is more likely that growth was stimulated by the inclusion of glutamine and/or tyrosine in FDM 4.

Skerman and Jayne-Williams (1966) found that a number of strains of C.bovis had a requirement for nicotinic acid, and others had no absolute requirement for any vitamin. In the present investigation C.bovis strain 124 had an absolute requirement for both riboflavin and thiamin with β-alanine, pyridoxine and biotin being stimulatory. The other strain, C.bovis strain 147, had more complex growth responses since although no growth occurred when no vitamins were included in the medium, a number of vitamins or other growth factors appeared capable of reciprocal replacement (Tables 20a and 20b). Biotin

however was demonstrated to have a stimulating effect upon growth (Table 21). Corynebacterium bovis also had an absolute requirement for glucose.

Growth occurred in the chemically defined media only when oleic acid or fatty acid-containing compound was included. In the case of a partially defined medium growth occurred when ovolecithin or synthetic β,γ-dipalmitoyl phosphatidylcholine was added. Due probably to the absence from the fully defined media of traces of substances capable of acting as solubilizers in the partially defined medium, the synthetic lecithins were not as efficiently dispersed in the fully defined media and it became necessary to use Tween 80 in a concentration of 0.005% to solubilize the synthetic lecithins. The lack of growth of the corynebacteria in the fully defined media to which only synthetic lecithin had been added could have been due either to the poor dispersion of the synthetic lecithins in the medium, or to the organisms being unable to effect the breakdown of the lecithin or the conversion of the fatty acid so obtained to the fatty acids specifically required. In this connection it should be noted also that the synthetic lecithins contained only palmitic acid, whereas ovolecithin contains both saturated and unsaturated fatty acids with 32% of the fatty acid being palmitic acid, and 43% being oleic acid (Romanoff and Romanoff, 1949). In order to determine which of these explanations was correct it would be necessary to find a method of achieving satisfactory dispersion of the synthetic lecithins without employing a surfactant as solubilizer, since the use of such a

surfactant obviously introduces yet a further modification to the medium. In the case of Tween 80 at the concentration used (0.005%), this did not alone support the growth of the organisms, although 0.05% Tween 80 allowed extremely good growth of C.bovis. However, the absence of growth in a medium containing 0.005% Tween 80 cannot allow the conclusion to be drawn that this concentration of Tween 80 does not have a stimulatory effect in the presence of the synthetic lecithin, possibly by providing oleic acid, or even by assisting the breakdown of the lecithin, or conversion of the palmitic acid. Such an effect has been suggested in the case of Tween 40 which has been used to detoxify oleic acid (Williams et al., 1947), but which has been considered by Hofmann (1963) to be capable of stimulating the biosynthesis of fatty acids. One method of solving the problem would be to effect the dispersion of the synthetic lecithins by the use of ultrasonic irradiation, a method found by Attwood and colleagues (1965) to give clear sols of aqueous dispersions of synthetic lecithins present in concentrations as high as 2% (w/v).

The finding that 0.05% (v/v) Tween 80 in the fully defined media allowed growth of the strains tested comparable to that obtained in tryptose serum broth agrees with the work of Cobb (1963, 1966) and Skerman and Jayne-Williams (1966). It seemed likely that the Tween 80 (or serum, ovolecithin or butter-fat) was fulfilling a requirement of C.bovis for fatty acid. However, it was just possible that these substances were affecting the growth of C.bovis by virtue of their surface-active properties. Since no facilities were

available for determining the surface tension of the completed media, an attempt was made to confirm or deny this latter hypothesis by substituting for Tween 80 other non-ionic surface-active agents possessing similar HLB (hydrophilic-lipophilic balance) values. Growth occurred in the presence of only a single surfactant, one in which the lipophilic moiety was formed from a glyceride oil, tending to confirm that the requirement was of a chemical rather than a physical nature.

In addition to growing on Tween 80 agar and in a fully defined medium to which Tween 80 had been added (e.g. FDM 5-9), C.bovis also grew on Tween 20 agar and in a fully defined medium to which Tween 20 had been added (FDM 5-24). Tween 20, however, does not exist as a pure polyoxyethylene sorbitan monolaurate, and it contains a substantial amount of oleic acid. Consequently the fatty acid requirement of C.bovis was most likely to be fulfilled by oleic acid. Microbial requirements for oleic acid (sometimes capable of being replaced by other unsaturated fatty acids) have been described by many workers including Cohen and his colleagues (1941), Hutner (1942), Williams and co-workers (Williams and Fieger, 1946; Williams et al., 1947), Pollock and colleagues (1949), Boughton and Pollock (1952), Deibel and Niven (1955), Morgan (1960), Johnson and Gary (1963), and Silliker and co-workers (1963). Niemen (1954) reviewed the work on fatty acid requirements of bacteria, observing that in general unsaturated fatty acids were growth-promoting, with saturated fatty acids being inactive, but fatty acids more unsaturated than oleic acid

exhibited less growth promotion because of their increasing anti-bacterial properties; *cis* fatty acids were generally more stimulatory than the *trans* isomers. In most cases where a micro-organism is found to have a requirement for oleic acid, this can be satisfied by the inclusion of Tween 80 in the medium. The advantage of Tween 80 is in providing a reservoir of oleic acid in a non-toxic form, but some organisms are so sensitive that they are inhibited by the small amounts of free fatty acid present in the medium. Under these circumstances this free fatty acid must be adsorbed by a substance such as bovine seralbumin fraction V which was found by Davis and Dubos (1947) to be necessary for the prevention of inhibition of *Mycobacterium tuberculosis* by free fatty acid present in the Tween 80 which was included in their media. When an oleic acid requirement is satisfied by the addition of oleic acid to the medium, the oleic acid must usually be rendered non-toxic since the concentration at which stimulation of growth occurs may be very close to the concentration at which inhibition of growth commences. Substances that have been used to render oleic acid non-toxic without affecting its growth-stimulating properties include bovine seralbumin fraction V, Tween 40, saponin and sodium taurocholate. In the present investigation it was found that oleic acid added to the fully defined basal medium would not allow growth and multiplication unless such a detoxifying agent was included. Although Tween 40 proved to be most satisfactory, its use presents some problems in the interpretation of the results since it also contains fatty acids and in addition Hofmann (1963) thought it capable of stimulating fatty acid

biosynthesis. The strains of C.bovis used were also able to grow in a fully defined medium containing oleic acid detoxified by the addition of albumin but morphological variation occurred. The concentration of oleic acid appeared to be fairly critical, reasonable growth being obtained in the presence of 10 mg oleic acid per litre of medium detoxified by the presence of 0.1% albumin whereas no growth occurred when the concentration of oleic acid was raised to 40 mg/l or lowered to 0.5 mg/l (the albumin content remaining unaltered). Thus the concentration of oleic acid at which growth occurred was 3.57×10^{-5}M, whereas no growth occurred when the concentration was dropped to 1.79×10^{-6}M or raised to 1.43×10^{-4}M. Hugo (1964) considered that a growth factor which was required at a level of 10^{-4} to 10^{-5}M for maximum growth was probably being incorporated into the cell, whereas for a growth factor to function as part of an enzyme system, the concentration for maximum growth could be as low as 10^{-8} to 10^{-10}M. It would appear that the oleic acid is actually being required by C.bovis in the synthesis of cell components and not acting as part of an enzyme system such as Deibel and Niven (1955) proposed in the case of the oleic acid requirement of the "minute" streptococci and Lactobacillus leichmannii.

Biotin appears to be involved frequently in the synthesis of unsaturated fatty acids, including oleic acid (Snell, 1951), and has been shown to replace the requirement of some bacteria for oleic acid (see for example Williams et al., 1947). The present investigation showed that in the case of C.bovis the inclusion of biotin in the medium did not replace the requirement for fatty acid although biotin

had a stimulatory effect when added to the medium in addition to a fatty acid source.

Deibel and Niven (1955) found that carbon dioxide (provided by incubating cultures in atmospheres containing concentrations of carbon dioxide above the normal atmospheric concentration) would replace the requirement of Lactobacillus leichmannii for an unsaturated fatty acid, although the oleate requirement of a pediococcus studied by Deibel, Silliker and Fagan (1964) could not be replaced by incubation under increased carbon dioxide tension. During the examination of the cultural requirements of C.bovis described in Section C, incubation in an atmosphere containing ca.5% carbon dioxide did not remove the necessity of adding serum, egg-yolk, butter-fat or Tween 80 to a medium such as tryptose lemco agar for growth to occur.

The optimum concentrations of the various essential constituents of the fully defined medium were they to be determined by the present system of batch culture would not necessarily reflect the requirements of the organism growing in its natural habitat. In the case of C.bovis its natural habitat - the bovine udder - provides an environment which would be more closely imitated in vitro by the use of a continuous culture technique, since in the udder the nutrients will be constantly renewed and the waste metabolic products of the bacteria together with a proportion of the bacterial growth will be continuously removed. In continuous culture the concentrations of metabolites required are much lower than the initial concentrations of metabolites required in batch culture to obtain a given increase in

cell mass or cell number. Continuous culture could also be used to determine the true oxygen requirements of the organisms and to determine whether carbon dioxide is required for growth from small inocula. It is probable that further investigation of the nutritional requirements of C.bovis by continuous culture techniques with the fully defined media already formulated providing the basis for modification may provide information concerning the reasons for the predilection of this organism for the bovine udder and its possible role in the initiation of bovine mastitis.

INVESTIGATION OF THE LIPASE OF C.BOVIS BY COLORIMETRIC AND ELECTROPHORETIC TECHNIQUES

AND ELECTROPHORETIC TECHNIQUES

Introduction

The reactions of C.bovis on tributyrin agar, Tween agars, butter-fat agar, Victoria blue margarine agar and Victoria blue butter-fat agar together indicate that C.bovis does possess lipolytic ability, although the reaction of the bacterium on any one medium provides evidence that is not conclusive, for the reasons put forward in Section D. It was thought that the in vitro examination of the enzyme or enzymes responsible might provide unequivocal evidence of the presence or absence of a lipase.

A lipase, if produced by C.bovis, would be either an extracellular enzyme or an enzyme bound to the cell membrane. If it were an extracellular enzyme then it would be possible to demonstrate its presence in culture filtrates. However if it were a membrane-bound enzyme it could only be extracted from the bacterium by disruption of the cells perhaps followed by separation of the enzyme from the cell membrane, either of these stages being likely to inactivate the enzyme. The absence of zone formation on butter-fat-or margarine-containing media with zone formation on Tween 20 agar and Tween 80 agar could be due to the production by C.bovis of a membrane-bound lipase and an extracellular Tween-hydrolysing enzyme (not necessarily a lipase, see page 63). The zones produced on tributyrin agar were always extremely narrow and always restricted to the top layers of the medium, but because of the inhibitory effect of the medium, the

interpretation of this reaction is rather difficult.

Thus it appeared that it should be possible to demonstrate the Tween-hydrolysing enzyme system in culture filtrates or in cell extracts. The demonstration of a true lipase (using water-insoluble substrates in a diphasic system) would be possible if it were identical with the Tween-hydrolysing enzyme but not if it were membrane-bound.

Whilst many studies have been made of extracellular lipase produced by bacteria (e.g. Alford, Pierce and Suggs, 1964) there have been few investigations of intracellular or membrane-bound lipases. Meyer, Malgras and Villiger (1962) extracted and purified a lipase from the Bacille-Calmette-Guérin strain of Mycobacterium tuberculosis by means of chromatography on a column of diethylaminoethyl cellulose.

In the present work it was decided to attempt the demonstration of lipase by two different methods: firstly, by use of a colorimetric method similar to that employed by Seligman and Nachlas (1950), and secondly by starch gel electrophoresis and polyacrylamide gel electrophoresis using a technique similar to that for the demonstration of esterases as used, for example, by Lawrence, Melnick and Weimer (1960).

Colorimetric techniques

Materials and Methods

Four typical strains of C.bovis (strain numbers 100, 119, 124, and 159) were chosen and each strain was grown in three different media - Tween 20 broth, Tween 80 broth and lecithin broth. The basal medium for these three broths was identical, consisting of (% w/v): tryptose (Oxoid L47), 1.0; Lab-Lemco beef extract (Oxoid), 0.3; sodium chloride, 0.5. The pH was adjusted to 7.2 and the medium distributed in 25 ml amounts in 100 ml Erlenmeyer flasks and sterilized by autoclaving at 121°C for 15 minutes. Tween 20 broth and Tween 80 broth were prepared by adding 0.02 ml of sterile Tween 20 or Tween 80 (sterilized by autoclaving at 121°C for 15 minutes) to each 25 ml amount of medium. Lecithin broth was prepared by adding aseptically 0.1 ml of a sterile 2% (w/v) ethanolic solution of lecithin (95 - 100% ovolecithin obtained from British Drug Houses Ltd., Poole, Dorset) to each 25 ml of medium. Sterilization of the lecithin solution was achieved by filtration through an asbestos filter (Carlson-Ford Grade EKS). The lecithin solution was added to the medium rapidly with the tip of the pipette below the surface of the medium, while the medium was swirled rapidly in the flask to help to achieve maximum dispersion of the lecithin. Inocula for the liquid media were obtained from 3 day serum agar cultures. After inoculation, broth cultures were incubated at 37°C, and cultures were removed for examination at intervals over a period of 12 days.

Preparation of extracts

Cell-free extracts were not prepared by filtration since filtration through asbestos filters has been shown to remove or inactivate microbial lipase (Hawkins and Stoenken, 1963). Extracts for examination were obtained by centrifuging each broth culture for 30 minutes at 4,000 r.p.m. The cell-free supernatant liquids so obtained were examined for the presence of extracellular lipase immediately after centrifuging.

The sediment of bacterial cells was examined for intracellular lipase after extracting the cells by the method of Kohn and Reis (1963). The bacterial sediment was washed twice with quarter-strength Ringer's solution and then a thick suspension made of the bacterial sediment in distilled water. One drop of chloroform was added for each millilitre of suspension, the mixture shaken in a tightly stoppered tube and stored for 2 days at $4^{o}C$. This was then centrifuged and the clear supernatant liquid examined for the presence of lipase. Kohn and Reis estimated that 90% of the enzyme activity of a bacterial suspension would be found in the supernatant liquid after such chloroform treatment, and they found that the extract could be kept, with chloroform as a preservative, at $4^{o}C$ for up to one year without loss of activity.

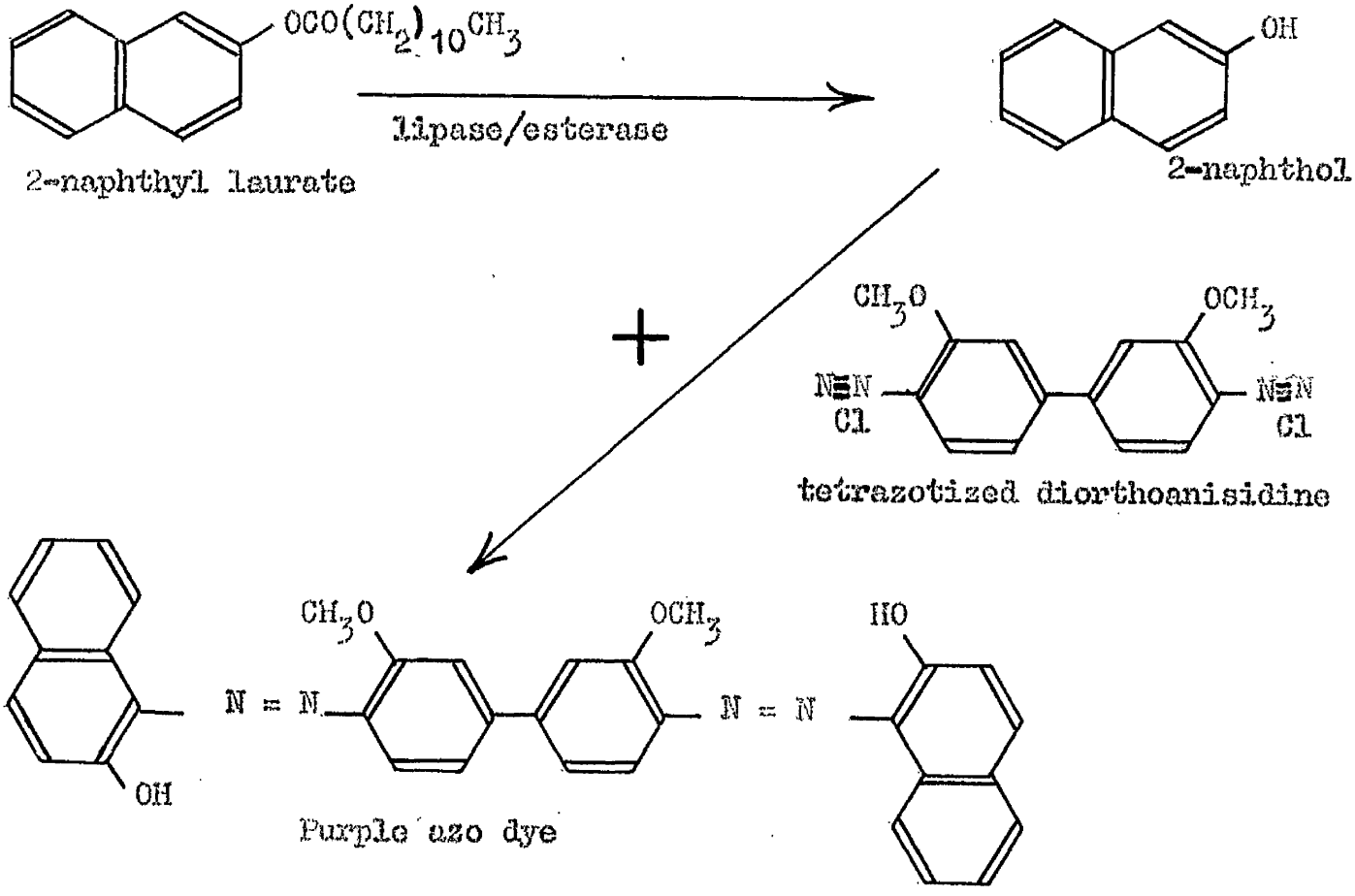
Examination of extracts

The extracts were examined for lipolytic activity by the method described by Nachlas and Seligman (1949), and Seligman and Nachlas (1950). The substrate consisted of 2-naphthyl laurate which was added,

as a solution in acetone, to an aqueous buffer solution to give an emulsion of the 2-naphthyl laurate. Since 2-naphthyl laurate is sparingly soluble in water, it will obviously be present in the aqueous phase as well as being present in the non-aqueous phase as a result of over-saturation. Desnuelle and Savary (1963) pointed out that such a substrate system may be acted upon by either lipase or esterase, the former hydrolysing the substrate in the non-aqueous phase, the latter hydrolysing the substrate in the aqueous phase. Consequently it is necessary to be able to differentiate between the activity of esterase and that of lipase. Differentiation between esterase and lipase has been obtained in the examination of tissue homogenates (Nachlas and Seligman, 1949) and serum (Seligman and Nachlas, 1950) by using sodium taurocholate to activate lipase (the esterase being slightly inhibited), and quinine hydrochloride to inhibit lipase (the esterase also being slightly inhibited). The effects of sodium taurocholate and quinine hydrochloride were therefore examined in the present work, although these compounds obviously may have different influences (if any) on bacterial enzymes.

The bacterial cell extracts were also examined using 2-naphthyl acetate as a substrate. Detection of the hydrolysis of the substrate depended upon the ability of the 2-naphthol released by enzyme activity to form a purple azo dye with tetrazotized diorthoanisidine (Fast Blue B Salt).

The purple azo dye thus formed is extractable with ethyl acetate, and the intensity of the colour of the extract is measurable



colorimetrically. An absolute measure of lipolytic activity can be found if required, by comparing the absorption values so obtained with a calibration curve prepared by the action of known concentrations of 2-naphthol on the Fast Blue B salt.

Substrate solution: consisted of 0.2 g of 2-naphthyl laurate or 2-naphthyl acetate (British Drug Houses Ltd.) dissolved in 100 ml of acetone, stored in the refrigerator for up to 1 month in a dark-glass bottle, after which it was discarded and fresh substrate solution prepared.

Buffer solution: consisted of 58 ml of a stock sodium diethyl barbiturate solution (10.3 g sodium diethyl barbiturate dissolved in

500 ml of distilled water), mixed with 42 ml of 0.1 M hydrochloric acid and 100 ml of distilled water. The prepared buffer solution was stored in a refrigerator for up to 1 month, after which it was discarded and fresh buffer solution prepared.

Calcium chloride solution: was prepared by dissolving 0.59 g hydrated calcium chloride ($CaCl_2.6H_2O$) in 100 ml distilled water.

Substrate-buffer solution: to 40 ml of buffer solution was added 1 ml of calcium chloride solution, followed by 5 ml of substrate solution. The substrate solution was added through a pipette with the tip of the pipette held beneath the surface of the buffer solution whilst the buffer solution was agitated. Finally 54 ml of distilled water was added. The resultant substrate-buffer solution was faintly opalescent.

Sodium taurocholate solution: was prepared by dissolving 0.537 g of sodium taurocholate in 100 ml of distilled water to give a 0.01 M solution. This was stored in the refrigerator until required, fresh solution being prepared every two months.

Treatment of extracts

One ml of broth culture supernatant liquid was placed in each of three test-tubes. To one tube was added 1 ml of sodium taurocholate solution, to the second tube was added 1 ml of quinine hydrochloride solution, and to the third tube was added 1 ml of distilled water. After allowing these tubes to stand for 10 minutes at 37°C, 5 ml of freshly prepared substrate-buffer solution was added to each tube. These mixtures were incubated for 2 hours at 37°C, after which 1 ml

of a freshly prepared 0.4% (w/v) solution of Fast Blue B salt (George T. Gurr Ltd., London, S.W.6) was added to each tube. This was allowed to react for 3 minutes and then 1 ml of a 40% (w/v) aqueous solution of trichloroacetic acid was added to the contents of each tube to stop enzymic action and facilitate the extraction of the dye. To extract the water-insoluble purple azo dye 10 ml of ethyl acetate were added to each tube which was then tightly stoppered with a rubber bung and vigorously shaken. The dye-containing ethyl acetate was separated from the aqueous phase by centrifugation, and 5 ml of the ethyl acetate transferred to a colorimeter tube. The intensity of the azo dye was determined with an EEL Model "B" absorptiometer (Evans Electroselenium Ltd., Halstead, Essex) using a No. 604 colour filter (peak transmission at a wavelength of 520 Å), this filter giving maximum sensitivity of the colorimeter to the azo dye.

In each case the zero reading of the instrument was adjusted using as a blank a reaction mixture identical except for the substitution of distilled water for the Fast Blue B salt solution. This therefore compensated for the appreciable light absorption of the appropriate medium and of the taurocholate solution (when used).

In order to determine the proportion of the light absorption in the reaction mixtures caused by free 2-naphthol that may have already been present in the substrate/buffer solution, control mixtures were used in which uninoculated broths replaced the culture supernatant liquid.

The chloroform-treated cell extracts were examined in a similar manner to the culture supernatant liquids using 2-naphthyl acetate and 2-naphthyl laurate as substrates. The small amounts of cell extracts available did not allow the effects of sodium taurocholate and quinine hydrochloride to be determined, and only 0.5 ml amounts of extracts could be used in each reaction mixture. Controls for the cell extracts were: 1) 2-naphthyl acetate/buffer solution, with distilled water in place of the cell extract, and 2) 2-naphthyl laurate/buffer solution, with distilled water in place of the cell extract.

Results

The optical density of a coloured solution measured with monochromatic light is proportional to the number of molecules absorbing the light, and it is therefore proportional to the concentration of the solute. In the EEL Model "B" absorptiometer used, the photo-electric cell and microammeter enabled a reading to be obtained of either the optical density of the solution under examination or the percentage transmission of light through the solution.

The narrow band spectrum filters in this instrument were inserted between the solution under test and the photo-electric cell. Thus the optimum filter for use with a given solution was the one which was complementary in colour to the solution, thus giving the highest density value and maximum sensitivity. This filter was shown to be No. 604 (peak transmission at a wavelength of 520 Å) in

the case of the azo dye being measured.

The first experiment consisted of tests on the four strains of C.bovis numbers 100, 119, 124 and 159, grown for 48 hours in Tween 80 broth and lecithin broth, and for 120 hours in Tween 20 broth (the longer incubation time for Tween 20 broth was chosen because of the relatively slow growth rate of the organisms in this medium). The activity of each broth culture supernatant liquid was determined with and without treatment with sodium taurocholate. The results are shown in Table 27.

The light absorption caused by the free 2-naphthol present in the substrate buffer solution as determined by the uninoculated control mixtures caused an optical density reading of 13.5 ~ 14.0. The O.D. values caused by release of 2-naphthol through the action of the culture extracts which are indicated in the tables were obtained by subtracting 13.5 from the colorimeter readings on the culture extracts.

Since increases in optical density caused by increases in the concentration of a coloured solute conform to Lambert's law and Beer's law, the colorimeter readings in Column 5 of table 27 can be adjusted, by substracting the reading obtained with the substrate/buffer control, to indicate the optical density value caused by release (assumed to be enzymic) of the 2-naphthol from the substrate. This value is shown in Column 6 of the table.

It can be seen that C.bovis strain 124 gave the greatest activity of the four strains tested, and therefore this strain was

TABLE 27. COLORIMETER READINGS ON BACTERIA-FREE BROTH CULTURES

Organism	2 Medium	3 Incubation time	4 Treated with taurocholate	5 Colorimeter reading	6 O.D. value caused by released 2-naphthol
C.bovis strain 100	Tween 20 broth	120 hours	No	22.5	9.0
			Yes	16.5	3.0
	" 80 "	48 "	Yes	22.5	9.0
	Lecithin "	48 "	No	15.5	2.0
			Yes	13.0	0
C.bovis strain 119	Tween 20 broth	120 hours	No	19.5	6.0
			Yes	13.0	0
	" 80 "	48 "	No	20.5	7.0
			Yes	23.5	10.5
	Lecithin "	48 "	No	13.5	0
			Yes	12.5	0
C.bovis strain 124	Tween 20 broth	120 hours	No	38.0	24.5
			Yes	35.5	22.0
	" 80 "	48 "	No	24.0	10.5
			Yes	30.5	17.0
	Lecithin "	48 "	No	13.5	0
			Yes	24.0	10.5
C.bovis strain 159	Tween 20 broth	120 hours	Yes	14.5	1.0
	" 80 "	48 "	No	20.0	6.5
			Yes	23.5	10.0
	Lecithin "	48 "	No	13.5	0
			Yes	12.0	0

selected for a further experiment in which the same three media were employed, with both broth culture supernatant liquids and cell extracts being tested several times over an incubation time of 283 hours in the case of Tween 20 broth and Tween 80 broth, and the cell extracts from the lecithin broth cultures being tested seven times during the period of incubation. The colorimeter readings obtained with the culture supernatant liquids are shown in Table 28, and those obtained with the cell extracts are shown in Table 29.

It should be noted that repeated readings of reaction mixtures gave O.D. values which could very by as much as 2.0 units on a reading of 20 (and proportionately more at higher O.D. values), that the O.D. values obtained for the 2-naphthol content of the substrate/buffer solution varied between 13.5 and 14.0 with the same batch of substrate/buffer solution, and that negative values of up to 2.0 units were obtained for culture activity after correction for the 2-naphthol content of the substrate. Thus the results should be regarded as qualitative only, the variability in readings being apparent even with the most careful attention to precision in preparing the reaction mixtures. Such factors as the warming-up time of the instrument, the length of time for which a tube was allowed to remain in the instrument, and voltage fluctuations in the electricity supply all caused considerable variations in the readings. Drift in the galvanometer deflection as a result of fatigue of the photoelectric cell was also observed. The influence of some, but not all, of these factors could be eliminated by very frequent checking and adjustment of the zero reading of the instrument.

TABLE 28. COLORIMETER READINGS OBTAINED WITH SUPERNATANT LIQUIDS FROM BROTH CULTURES OF C.BOVIS STRAIN 124

Medium	Treatment	Colorimeter reading after incubation of cultures for the following times (hours)						Optical density value caused by release of 2-naphthol					
		19	43	68	115	163	283	19h	43h	68h	115h	163h	283h
Tween 20	None	17.75	14.5	11.75	12.8	15.5	-	4.25	1.0	-1.75	-0.7	2.0	-
	Taurocholate	16.0	16.2	17.6	13.5	13.5	15.5	2.5	2.7	4.1	0	0	2.0
	Quinine	12.3	14.6	11.5	12.6	15.5	14.0	-1.2	1.1	-2.0	-0.9	2.0	0.5
Tween 80	None	16.5	16.3	15.25	18.3	15.75	14.25	3.0	2.8	1.75	4.8	2.25	0.75
	Taurocholate	13.3	16.6	16.2	20.5	21.5	14.75	-0.2	3.1	2.7	7.0	8.0	1.25
	Quinine	14.8	15.5	13.5	14.7	13.75	11.5	1.3	2.0	0	1.2	0.25	-2.0

TABLE 28. COLORIMETER READINGS OBTAINED WITH SUPERNATANT LIQUIDS FROM BROTH CULTURES OF C. BOVIS STRAIN L24

TABLE 29. COLORIMETER READINGS OBTAINED WITH CELL EXTRACTS FROM BROTH CULTURES OF C.BOVIS STRAIN 124

Medium	Incubation time (hours)	Colorimeter readings obtained with:			Optical density value caused by release of:		
		2-Naphthyl acetate	2-Naphthyl laurate	2-Naphthyl laurate after treatment with taurocholate	2-Naphthyl acetate	2-Naphthyl laurate	2-Naphthyl laurate after treatment with taurocholate
Tween 20 broth	19	8.5	16.5	-	4.5	2.5	-
	43	10.0	14.5	-	6.0	0.5	-
	68	9.0	16.0	-	5.0	2.0	-
	115	-	-	64.0	-	-	20.0
	163	-	24.0	62.0	-	10.0	18.0
	211	-	18.0	58.0	-	4.0	14.0
	283	-	13.75	59.0	-	-0.25	15.0
Tween 80 broth	19	9.0	16.5	-	5.0	2.5	-
	43	9.0	17.5	-	5.0	3.5	-
	68	9.0	16.5	-	5.0	2.5	-
	115	-	21.5	62.0	-	7.5	18.0
	163	-	17.4	70.0	-	3.4	26.0
	211	-	20.0	58.0	-	6.0	14.0
	283	-	20.0	60.0	-	6.0	16.0
Lecithin broth	19	8.5	13.5	-	4.5	-0.5	-
	43	12.0	16.5	-	8.0	+2.5	-
	68	15.0	21.0	-	11.0	7.0	-
	115	-	20.0	66.0	-	6.0	22.0
	163	-	18.0	66.0	-	4.0	22.0
	211	-	19.25	56.0	-	5.25	12.0
	283	-	20.0	57.0	-	6.0	13.0
Substrate/ buffer + Fast Blue B salt		4.0	14.0	44.0			

TABLE 29: COLORIMETER READINGS OBTAINED WITH CELL EXTRACTS FROM BROTH CULTURES OF C.BOVIS STRAIN 124

Electrophoretic techniques

Materials and methods

Two basic electrophoretic techniques were employed - in the first starch-gel was the supporting medium, a technique first described by Smithies (1955), and in the second the supporting medium was polyacrylamide gel, the use of which was described by Raymond and Wang (1960).

Cultures

Both broth and agar cultures were employed. Broth cultures were used to examine both supernatant liquids and cell extracts. One hundred-ml Erlenmeyer flasks containing 25 ml amounts of Tween 80 broth were inoculated from 3 day serum agar cultures and incubated at 37°C for 3 days. The Tween 80 broth consisted of Bacto-Casitone (Difco), 5.0 g; tryptose (Oxoid), 5.0 g; Lab-Lemco beef extract (Oxoid), 3.0 g; sodium chloride, 5.0 g; distilled water, 1 litre; pH 7.2; sterilized by autoclaving at 121°C for 15 minutes, and sterile Tween 80 added aseptically to a final concentration of 1% v/v.

Agar cultures were prepared by growing the bacteria on 200 ml quantities of 5% serum agar in 1 litre Roux culture flasks. The medium was inoculated from 3 day serum agar cultures by preparing a suspension of the corynebacteria in sterile ¼-strength Ringer's solution and aseptically spreading 0.5 ml of this suspension over the surface of the agar in the Roux culture flasks. Cultures were incubated for 3 days at 37°C.

Preparation of extracts from broth cultures

Broth cultures of C.bovis strains 119, 124 and 147 were

centrifuged after incubation for one, two and three days at 37°C. The bacterium-free supernatant liquids were used as samples both before and after concentration by ultrafiltration through collodion membranes. The ultrafiltration was carried out under negative pressure in the Membranfilter ultrafiltration apparatus (manufactured by Membran filter Gesellschaft, Gottingen, and distributed by Hudes Merchandising Corp., Ltd., 52, Gloucester Place, London, W.1). Before ultrafiltration the apparatus was placed in a refrigerator until thoroughly chilled. During ultrafiltration, the apparatus was packed round with crushed ice. Ultrafiltration for 3 hours concentrated the sample four to eight times. The bacterial sediments from the broth cultures obtained by centrifugation were washed twice in sterile ¼-strength Ringer's solution and suspended in a small amount of distilled water to produce a thick creamy suspension. The cells were extracted by the method of Kohn and Reis (1963), by adding chloroform (1 drop for each ml of suspension) to each bacterial suspension, shaking the mixture in a tightly stoppered tube and refrigerating at 4°C for 2 days.

Preparation of extracts from agar cultures

The bacterial growth was harvested from each Roux culture flask by washing with 10 ml of sterile ¼-strength Ringer's solution. This suspension was centrifuged and the bacterial sediment washed twice with sterile ¼-strength Ringer's solution. The bacterial sediments were extracted by the following methods.

a) Grinding with alumina as described by McIlwain (1948).

Approximately equal amounts of packed bacterial sediment and Grade 3/50 slow-cutting polishing alumina (obtained from Griffin and George Ltd., Braeview Place, East Kilbride) were mixed and placed in a tissue-grinding tube. The mixture was chilled thoroughly by placing the tissue-grinding tube in a beaker of crushed ice and water. The mixture was ground for about 3 minutes until it had ceased to be dry and had become a moist cream. During the grinding operation the tissue-grinding tube was retained in the beaker of crushed ice and water. After grinding, about 1 ml of the appropriate buffer solution (that is, the same buffer solution as was used in the preparation of the starch gel or polyacrylamide gel) was mixed with the paste and then centrifuged. The supernatant liquid was retained refrigerated in rubber-stoppered 2 x $\frac{1}{4}$-inch tubes until required for electrophoresis. Electrophoresis was carried out within 2 hours of the preparation of the extract.

b) Acetone drying, as described by Umbreit, Burris and Stauffer (1957). The harvested and washed bacterial growth was prepared as a thick cream in $\frac{1}{4}$-strength Ringer's solution and added slowly to 10 volumes of ice-cold dry acetone with constant stirring. After stirring for 5-10 minutes, the bacterial cells were allowed to settle and the sediment filtered by suction through a cellulose acetate membrane filter (Oxoid Ltd. London, S.E.1). The residue was washed with a small amount of dry cold acetone, which was removed rapidly by suction. The residue was dried over silica gel in an evacuated desiccator which was kept in the refrigerator until the residue was

dry. When required for electrophoresis the dry powder was suspended in a small amount of a buffer solution of the same constitution as that used for preparing the electrophoresis gel.

c) Acetone drying, followed by grinding with alumina. Since Umbreit and colleagues (1957) found that acetone-dried preparations sometimes did not give cell-free enzymes even after suspending in a buffer solution, it was decided to examine the effect of following the acetone-drying described in (b) by grinding with alumina as described in (a). For this purpose a small amount of a buffer solution appropriate to the electrophoresis system being used (see below) was added to the acetone-dried powder to give a thick, creamy suspension which was ground after adding an equal amount of alumina, as described in (a).

d) Ultrasonic disintegration. The bacterial cells were suspended in buffer solution to give a liquid of creamy consistency. The cells were disintegrated by exposing them to 10 minutes vibration from an M.S.E. Ultrasonic disintegrator tuned for maximum output. During the treatment the glass cell containing the bacterial suspension was immersed in crushed ice and water to minimize the effect of heating.

e) High frequency shaking with glass beads. To a creamy suspension of bacterial growth in buffer solution was added an equal quantity of acid- and water-washed ballotini (English Glass Co. Ltd., Leicester). This mixture was subjected to 5 minutes of high-frequency shaking in a Braun disintegrator in which solid carbon dioxide was generated to cool the suspension during the disintegration.

Lipase standard

Since no description of a method for demonstrating lipase after zone electrophoresis had been found when this work was undertaken, it was necessary to examine possible methods for detecting lipase using as a standard a sample known to possess lipolytic powers. The standard chosen was wheat germ lipase (stated activity 60 U per mg, obtained from Koch-Light Laboratories, Colnbrook, Bucks.) suspended in tris/citrate buffer (see below). The wheat germ lipase was examined both with and without grinding with alumina(grinding method as described on page 139).

Buffer solutions and lipase-detecting systems

In order to ascertain the compatibility of the buffer solution to be used during electrophoresis and the enzyme-detecting system to be used after completion of electrophoresis, a number of possible lipase-detecting systems were tested in the presence of varying buffer solutions. These tests were carried out with and without the addition of wheat lipase (Koch-Light Laboratories, Colnbrook, Bucks). The colour reaction or precipitate obtained in the presence of lipase was compared with a control containing no lipase.

The systems examined were as follows.

Substrate	Detection
2-naphthyl laurate	Fast Blue B salt
Tween-containing overlays	Precipitation of calcium salts of free fatty acids
Triolein, Tributyrin, Ethyl oleate, Olive oil	Nile blue sulphate or p-aminodimethylaniline hydrochloride.

Three different buffer solutions were examined:

a) Tris-maleate buffer solution (Gomori, 1952) consisted of maleic acid, 29 g and 2-amino-2-(hydroxymethyl)-propane-1:3-diol (tris buffer), 30.3 g dissolved in 500 ml of distilled water. To this was added 2 g of powdered charcoal after which the mixture was shaken and allowed to stand for 10 minutes before the charcoal was filtered off. To 40 ml of this solution was added N sodium hydroxide (the amount of which depended on the required pH of the buffer solution as indicated below), and the mixture diluted to 100 ml with distilled water.

N NaOH required (ml) :	10.5	16.5	19.0	22.5	26.0
pH of buffer obtained :	6.05	6.9	7.65	8.1	8.7

b) Barbital buffer solution (Gomori, 1952) consisted of barbital sodium, 1.03 g, dissolved in 50 ml of distilled water, to which was added 0.1 N hydrochloric acid (the amount of which depended on the required pH of the buffer solution as indicated below), and the mixture diluted to a total volume of 100 ml with distilled water.

0.1 N HCl required (ml) :	41.0	31.0	19.0	7.5
pH of buffer obtained :	7.55	7.95	8.25	8.8

c) Tris buffer solution (Gomori, 1952) consisted of 2-amino-2-(hydroxymethyl)-propane- 1:3-diol (tris buffer), 2.1 g, in 50 ml of

distilled water, to which was added N hydrochloric acid (the amount of which depended on the required pH of the buffer solution as indicated below), and the mixture diluted to a total volume of 100 ml with distilled water.

N HCl (ml)	: 16.0	13.5	9.0
pH of buffer obtained	: 7.3	7.75	8.2

Tris-maleate buffer solution has been used for electrophoresis of esterases by Lawrence, Melnick and Weimer (1960), and a barbital sodium buffer was used by Sarda and Desnuelle (1958) in the preparative zone electrophoresis of lipase and esterase on starch columns.

The five substrate preparations examined were:

1) Triolein, tributyrin and ethyl oleate emulsions. Polyvinyl alcohol was used as an emulsifier as described by Fiore and Nord (1949). Polyvinyl alcohol, 10.0 g, was stirred into 1 litre of distilled water for 30 minutes using a magnetic stirrer. Five ml of 0.1 N hydrochloric acid were added and the mixture heated to 80°C with stirring, and held at 80°C in a water bath until the polyvinyl alcohol had completely dissolved. One hundred ml of distilled water were added, the mixture heated for a further hour, after which it was cooled, filtered, and adjusted to pH 7.0 with 0.1 N sodium hydroxide. To prepare the emulsion 50 ml of triolein, tributyrin or ethyl oleate were added to 100 ml of polyvinyl alcohol, and

homogenized for 5 minutes in an Atomixer (M.S.E. Ltd., London, S.W.1). The prepared emulsion was kept refrigerated until required, and shaken vigorously before pipetting.

2) Sigma Stabilized Olive Oil Emulsion (Sigma Chemical Co. Ltd., London).

3) 2-naphthyl laurate, 2% (w/v), in acetone.

The reaction mixtures were prepared in the case of the triolein, tributyrin or ethyl oleate emulsions by mixing 5 ml of substrate/polyvinyl alcohol emulsion, 5 ml of buffer solution and 5 ml of lipase solution (wheat lipase, 0.1% w/v, in distilled water). The reaction mixture containing 2-naphthyl laurate as the substrate was prepared by pipetting 0.1 ml of the 2-naphthyl laurate solution under the surface of 9.9 ml of buffer solution with constant agitation, after which 5 ml of lipase solutionwere added. Control tubes consisted of mixtures in which 5 ml of distilled water had been substituted for the enzyme preparation.

After incubation overnight the mixtures containing triolein, tributyrin, ethyl oleate or olive oil as substrate were examined either by addition of 2 ml of a freshly prepared 0.5% (w/v) solution of p-aminodimethylaniline hydrochloride (after Castell and Bryant, 1939; Castell, 1941), or by addition of 2 ml of an aqueous solution (made alkaline with 0.1N sodium hydroxide) of nile blue sulphate (prepared by diluting 5 ml of a proprietary concentrated nile blue sulphate solution of unknown concentration to 200 ml with distilled water).

The preferred lipase-detecting system giving the maximum colour

differentiation between the mixture containing lipase and the control mixture containing no lipase, was found to be 2-naphthyl laurate/Fast Blue B salt with the best colour differentiation occurring in buffer solutions below pH 8 (above pH 8 the control mixture tended to be quite darkly coloured). The barbital buffer at pH 7.55 gave the deepest colour reaction with the lipase. Consequently barbital buffer at pH 7.55 was one of the buffers used in carrying out the electrophoresis, this buffer being employed both in the preparation of the gel and as the buffer solution in the electrophoresis tank compartments.

In addition, the discontinuous buffer system first used by Poulik (1957) was also employed, in the concentration recommended by Baillie and Norris (1963). In Poulik's discontinuous buffer system, the gel is prepared in a tris-citric acid buffer and the electrode compartments of the electrophoresis tank contain a boric acid buffer. Such a system was found by Poulik to give a sharper separation of compounds, with the edges of the zones becoming more clearly defined as the boundary between the buffers moves across them. In addition migration occurred more rapidly in a discontinuous buffer system, Poulik considering that this was probably due to the voltage changes that occurred across the moving boundary between the buffers. Another advantage of this discontinuous buffer system is that the boundary is visible to the unaided eye as a thin brown line, so the progress of the electrophoresis can be followed. A stock solution of 2-amino-2-(hydroxymethyl)-propane-1:3-diol (tris buffer),

18.36 g, citric acid, 2.12 g, made up with distilled water to 1 litre was prepared. For a working solution in which a gel was to be prepared, 30 ml of stock solution were diluted with 90 ml of distilled water. The tank buffer consisted of boric acid, 18.5 g, sodium hydroxide, 2.0 g, made up with distilled water to 1 litre.

Both the buffer systems chosen were theoretically suitable for the electrophoresis of macromolecular solutes (Morris and Morris, 1964) since both the tris buffer and the barbital sodium were large organic ions with low mobilities, a property which tends to minimize conductance changes at the zone boundaries (the so-called "boundary anomalies"). In addition the tris buffer was cationic and the barbital anionic; thus in using these two buffers both main types of buffer suitable for electrophoresis of macromolecules were represented.

Preparation of gels

Gels were poured into Perspex trays consisting of a 7" x 4½" base with removable side pieces ½" wide by ½" deep bolted on, each gel thus being 6" x 3½" x ½". Two such gels on their base plates could be accommodated side by side in the electrophoresis tank.

Before pouring the gels, the inside surfaces of each gel tray were thinly coated with silicone by brushing with a dispersion of silicone grease (M494 grease manufactured by I.C.I. Ltd., Stevenston, Ayrshire) in acetone, and allowing the acetone to evaporate.

Starch gels of the type first described by Smithies (1955) were prepared by the method of Smith (1960). To 120 ml of buffer

solution (either 30 ml of stock tris-citric acid solution diluted with 90 ml of distilled water, or the 0.05 M barbital pH 7.55 buffer described on page 143) were added 12.24 g of hydrolysed starch, batch 204-1 (manufactured by Connaught Medical Laboratories, Toronto, Canada and obtained from Arnold R. Horwell, 17, Cricklewood Broadway, London, N.W.2). The concentration of hydrolysed starch used was that recommended by Connaught Laboratories for this batch. The starch was added to the buffer solution in a 500 ml conical flask of borosilicate glass, and mixed to a completely even suspension by vigorous swirling. Constant swirling was maintained while the flask was heated carefully over a fairly small Bunsen flame. Care was taken that the suspension was heated evenly and not too rapidly. The suspension gradually thickened to a viscous, semi-solid, opaque mass, heating and vigorous swirling being continued until the mixture became a translucent but still fairly viscous liquid. When this stage was reached heating was discontinued and a negative pressure immediately applied from a water pump. The liquid was allowed to boil under negative pressure for exactly one minute. The starch was then immediately poured into the Perspex tray on a levelling board until the surface of the gel was just above the level of the sides of the tray. A sheet of Melinex Type O polyethylene terephthalate film (I.C.I. Ltd., Welwyn Garden City, Herts.) was carefully placed on the surface of the gel with a rolling action in order to exclude all air bubbles. The starch gel was allowed to set for exactly two hours at room temperature. The gels were always used when prepared and were never stored before use.

Polyacrylamide gels were prepared by the technique of Raymond and Wang (1960). In order to provide sample slits in the gels, Perspex lids to the trays were employed instead of the Melinex film used in the case of starch gels, each lid carrying two Perspex formers 1" wide by $\frac{1}{4}$" deep by 1/16" thick. Thus in each gel tray two samples could be run from slits 1" wide and the full depth of the gel. In the case of polyacrylamide gels, only the discontinuous buffer system of Poulik (1957) was employed, with the gel buffer being used at the same concentration as that used by Poulik, that is 60 ml of stock tris-citric acid solution were mixed with 60 ml of distilled water. In 120 ml of buffer solution, 8.4 g of Cyanogum 41 (B.D.H. Ltd., Poole, Dorset) were dissolved, after which 2.4 ml of a 1% (w/v) solution of ammonium persulphate were added and well mixed, followed by 2.4 ml of a 1% (w/v) solution of dimethylaminopropyl cyanide. During this procedure care was taken not to introduce air bubbles, and the distilled water used to dilute the stock buffer solution had been previously boiled to expel dissolved air, since the polymerization of the Cyanogum 41 was inhibited by the presence of oxygen. Immediately after the addition of the reagents, the mixture was poured into a Perspex gel tray (the same type as used for making starch gels) which, in addition to being coated with silicone as previously, also had the joints between the removable sides and between the sides and the base sealed with silicone grease to prevent the entry of air. Sufficient mixture was added to stand proud of the edges of the tray. The Perspex lid incorporating the sample

slit formers was then lowered into position taking care to exclude all air bubbles. The prepared tray was left undisturbed until the gel had set (about 30 minutes) after which the gel in its tray was chilled in a refrigerator.

Application of samples

Samples were applied to the starch gels with the aid of filter paper. The removable short sides of the gel tray were removed and a straight cut made with a razor blade across the width of the gel $1\frac{1}{2}$" from one end. The short end was pushed carefully back to reveal the cut surface. A piece of filter paper (Whatman Grade 3 MM) $\frac{1}{4}$ x $\frac{3}{8}$" was held in forceps and saturated with the sample, after which it was touched to a piece of filter paper to remove all excess liquid. The filter paper piece carrying the sample was carefully placed on the cut face of the longer piece of gel taking care to exclude all air-bubbles. Up to five samples could be run on each gel with $\frac{1}{4}$" space between adjacent samples. After the samples had been placed in position, the short end of the starch gel was pushed back into place, once again care being taken to exclude all air bubbles, and the Melinex film was replaced on the gel.

In the case of the polyacrylamide gels the samples were placed in the preformed sample slits using finely drawn pasteur pipettes. Before the samples were placed in the slits it was necessary first to seal the base of the slits at the gel/Perspex junctions with molten vaseline using a pasteur pipette, the Vaseline solidifying on contact with the pre-chilled gel and base plate. The sample

slits were filled to the top (but not above the top) with liquid sample and the slits sealed with molten Vaseline which immediately solidified. The surface of the polyacrylamide gel was then covered with Melinex film.

In both starch gel and polyacrylamide gel electrophoresis, one of the samples on each gel consisted of a bovine serum reference standard. This standard serum originated from a single batch of bovine serum (Oxoid), and was dispensed in 2 x $\frac{1}{2}$ inch rubber stoppered tubes and held deep frozen at - 15°C. A tube of serum was defrosted immediately before use and once melted was discarded and not refrozen.

Electrophoresis

The gel trays were placed in a Shandon Universal Electrophoresis Tank Kohn type Mark II (Shandon Scientific Co. Ltd., 65, Pound Lane, Willesden, London, N.W.10), the appropriate buffer solutions having already been placed in the electrode compartments and the space between the double walls of the tank having been filled with crushed ice to minimize the temperature rise during the run. Contact between the ends of the gels and the electrode compartments was made by wicks made of white absorbent lint which extended the full width of the gels. The gels, and the wicks at the points of contact with the gels, were covered with Melinex film to prevent evaporation of water from the gels and the wicks and to protect the gels from condensate falling on them.

The stabilized electricity supply was obtained from an A.E.I. two channel stabilized power supply unit Type R 1280 (manufactured

by Associated Electrical Industries Ltd., Telecommunications Division, Woolwich, London, S.E. 18), which gave a supply at a chosen constant voltage. A constant current supply was unobtainable from this apparatus. The output voltage of the power supply was adjusted to give a potential drop of 8 volts per cm across the gel, as judged by an Avometer with probes placed in the tank buffer compartments. In the case of electrophoresis with a discontinuous buffer system (the buffer system most frequently used) the migration of the buffer boundary could be observed, and the electrophoresis was continued until the brownish buffer boundary had reached a point 10 cm from the sample slits. In the case of continuous buffer systems, the progress of electrophoresis was followed by prestaining the serum sample used as a reference standard with bromphenol blue which caused the albumin component of the serum to be coloured blue without affecting its mobility (Raymond and Wang, 1960).

The time required for electrophoresis was usually five hours.

Slicing of gels after electrophoresis

Each gel was cut into four 1/16" thick slices using a coping saw in which the blade had been replaced by a taut stainless steel wire (a banjo A string). Slicing of the gels was achieved by removing the long sides of the gel tray and replacing each of them with a stack of three 1/16" thick Perspex strips, which were used as guides to cut the first slice. This slice was removed by rolling it off still attached to the Melinex film. One Perspex strip was removed from each side, and a second 1/16" thick slice was cut and

also removed by applying a piece of Melinex film, and rolling off the gel slice which adhered to the film. In a similar way the third and fourth slices of gel were obtained. The first slice of gel was of slightly uneven thickness in the case of starch gels and it was therefore discarded. The three slices of each starch gel and four slices of each polyacrylamide gel thus obtained were examined by one or more of the techniques described below. Staining procedures involving liquid reagents were carried out at room temperature unless otherwise stated in 10 x 8" plastic boxes fitted with air-tight lids to prevent evaporation of solvents. Techniques involving overlay of the gel with a solution or emulsion of the reagent solidified with agar are described in full below.

Staining for proteins

The effectiveness of each of the staining solutions recommended by Bodman (1960) was examined, in addition to the protein stain of Fischl & Gabor (1963).

a) The gels were stained for 30 seconds in a 1% solution of naphthalene black 10B or naphthalene black TS (both stains obtained from George T. Gurr, London) in a mixture of 50 parts methanol, 50 parts of distilled water, 20 parts of glacial acetic acid. Decolorization was achieved in four changes of the same solvent, the gel being left overnight in the last wash (Bodman, 1960).

b) The gels were stained for 30 seconds in a 0.1% (w/v) solution of naphthalene black 10B or naphthalene black TS in a sodium acetate/acetic acid mixture (1 part of a 0.1M sodium acetate

solution to 1 part of M acetic acid). Decolorization was carried out in a number of washes in 5% acetic acid, with the gel remaining in the last wash for 24 hours (Bodman, 1960).

c) The gels were stained in a 0.02% solution of nigrosin in a mixture of ethanol, trichloroacetic acid and water. The working nigrosin solution was prepared by dissolving 0.5 g of nigrosin in a mixture of 95% ethanol, 200 ml, 25% trichloroacetic acid, 50 ml, distilled water to 500 ml, followed by filtration and dilution of 1 part of the stock nigrosin solution with 4 parts of the same solvent mixture. Staining of the gels was carried out overnight in an air-tight box, after which they were decolorized overnight in a 5% solution of trichloroacetic acid (Bodman, 1960).

d) Staining of the gel was carried out in the trichrome stain of Fischl and Gabor (1963). This staining solution consisted of equal amounts of (i) a 0.2% solution of Light Green (George T. Gurr) in 0.5% sulphosalicylic acid, (ii) a 0.2% solution of Ponceau Red (George T. Gurr) in 5% trichloroacetic acid, and (iii) a 0.1% solution of naphthalene black in 5% trichloroacetic acid. The gels were stained for five minutes and then decolorized in a number of changes of 5% acetic acid until only a faint background colour remained.

Of these staining methods nigrosin gave the clearest results in starch gels and naphthalene black in sodium acetate/acetic acid solution was preferred for staining polyacrylamide gels. The results shown in Figures 7-11 were therefore obtained using [illegible]

the latter stain.

Staining for catalase

The gel slice was flooded with a 5% solution of 20-volume hydrogen peroxide for 30 seconds, followed by rinsing with water. Catalase bands caused the evolution of oxygen as numerous small bubbles. The position and width of the bands were measured.

Staining for esterase

The method of Lawrence, Melnick and Weimer (1960) was employed. The gel slice was flooded with the following solution: 0.1M 2-amino-2-(hydroxymethyl)-propane-1:3-diol, 30 ml; 0.1M maleic acid, 20 ml; 1% 1-naphthyl acetate in 50% acetone, 2 ml; Fast Blue B salt 25 mg. The solution was prepared immediately before use by adding the 1-naphthyl acetate solution to the buffer with constant mixing, the tip of the pipette being held below the surface of the liquid, after which the Fast Blue B salt was dissolved in the mixture. The solution was allowed to act on the gel slice for 1 hour at room temperature. Esterase bands developed as reddish-purple areas. If the esterase bands were not well-developed after 1 hour, it was found that it was possible to obtain deeper staining of the bands without excessive background staining by allowing the gel slices to remain in the developing solution for even as long as 18 hours. After overnight incubation gentle rinsing of the gels removed any deposit that had formed on the gels.

Staining for lipase

The method employed was similar to that used for the detection

of esterase, but the substrate was 2-naphthyl laurate instead of 1-naphthyl acetate. The Fast Blue B salt used was able to react with 2-naphthol as well as 1-naphthol to form purple azo dyes.

Demonstration of lipase with olive oil as the substrate

The gel slice to be examined was placed on a piece of Melinex film on a sheet of Perspex. To 10 ml of molten 3% agar (Oxoid Agar No. 3, 3 g, in 100 ml of distilled water) were added at 45°C 5 ml of Sigma Stabilized Olive Oil Emulsion. After mixing this was poured quickly over the gel slice and allowed to set as a thin layer. Melinex film was then placed on the surface of the agar. The prepared gel slice was incubated overnight at 37°C or at room temperature and then examined for clearing or precipitation. In the absence of any reaction, incubation was continued for another 48 hours.

Trials not involving electrophoresis in which the action of wheat lipase on the solidified olive oil was examined revealed that a zone of granular precipitation occurred which was similar to that developing in Tween agar as a result of bacterial action: the coarsely granular precipitate was accompanied by a decrease in the background turbidity in the zone, so that the reaction was clearly visible.

Demonstration of lipase (Tween-hydrolysing enzymes)

To 10 ml of molten 3% agar (Oxoid Agar No.3) containing 0.1% calcium chloride were added 10 ml of 2% Tween 20, Tween 40, Tween 60 or Tween 80. After mixing this was poured quickly over the gel

slice to be examined, which had previously been placed on a piece of Melinex film. The Tween/agar mixture was allowed to set as a thin layer and it was covered with a piece of Melinex film. The prepared gel slice was incubated overnight at 37°C or at room temperature, and then examined for the presence of precipitated calcium salts of fatty acids. In the absence of a reaction, incubation was continued for a further 48 hours.

Results

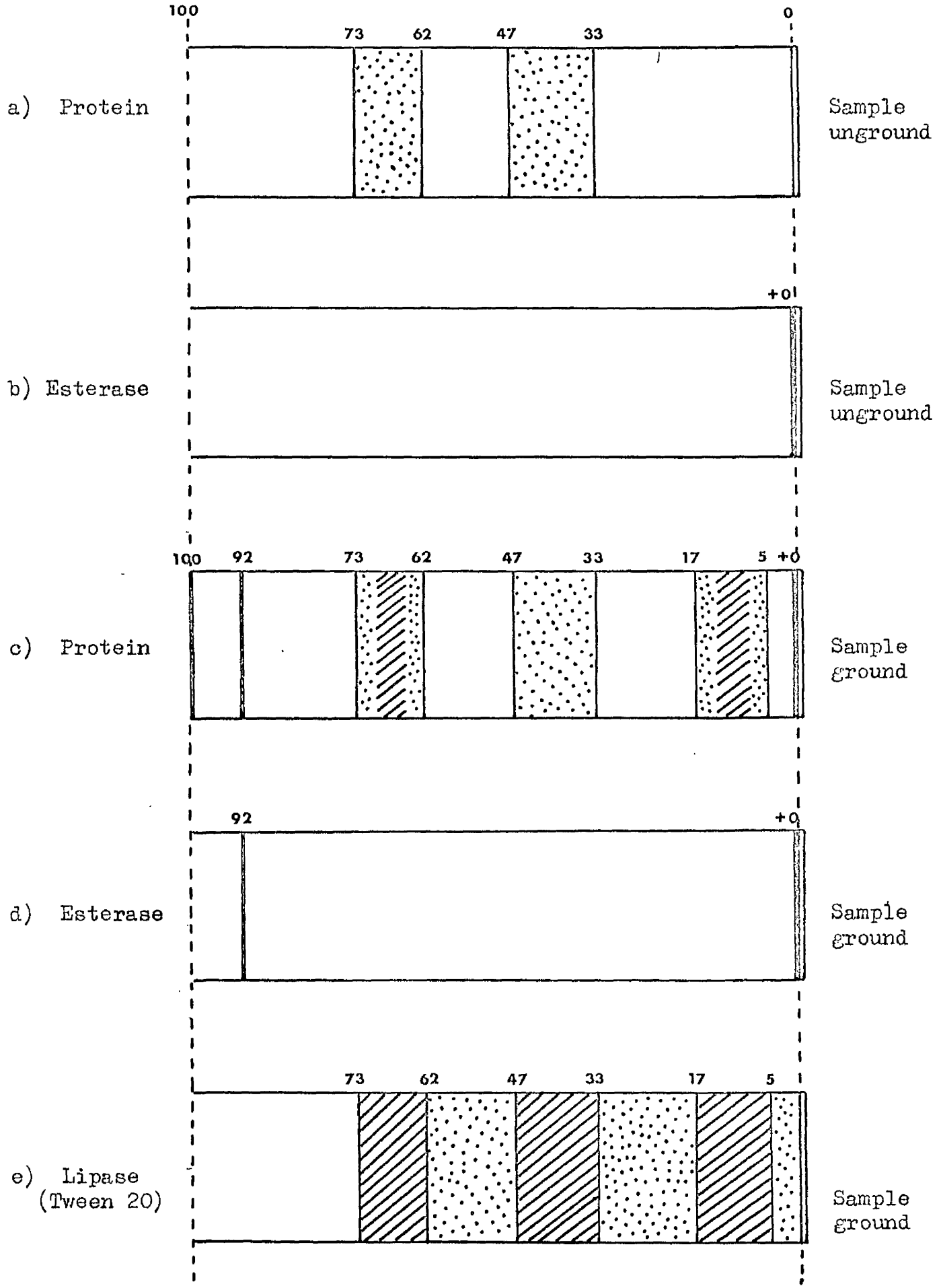
The effectiveness of the techniques used for staining protein and for detecting esterase and lipase was checked using wheat lipase suspensions both before and after grinding. The results of this are shown in Figure 7. An unground wheat lipase sample gave two protein bands (Fig. 7a) and an esterase band at the leading edge of the sample slit (Fig. 7b).

No reaction was obtained with naphthyl laurate. Precipitation was obtained with Tween 20, Tween 40 and Tween 60 at the sample origin only, no reaction occurring with Tween 80 or with olive oil.

The alumina-ground sample of wheat lipase showed an additional four protein bands, and one of the two bands found in the unground sample was intensified (Fig. 7c). Two esterase bands were discerned in the case of the ground sample - one at the front edge of the sample slit as before, and the second was a fast-moving band that occurred only a short distance behind the buffer boundary (Fig. 7d). The mobile esterase band corresponded with a protein band. The ground wheat lipase was found to give no reaction with naphthyl

FIGURE 7. PROTEIN, ESTERASE AND LIPASE ELECTROPHORETOGRAMS OF WHEAT LIPASE.

(Polyacrylamide gel / discontinuous buffer system)

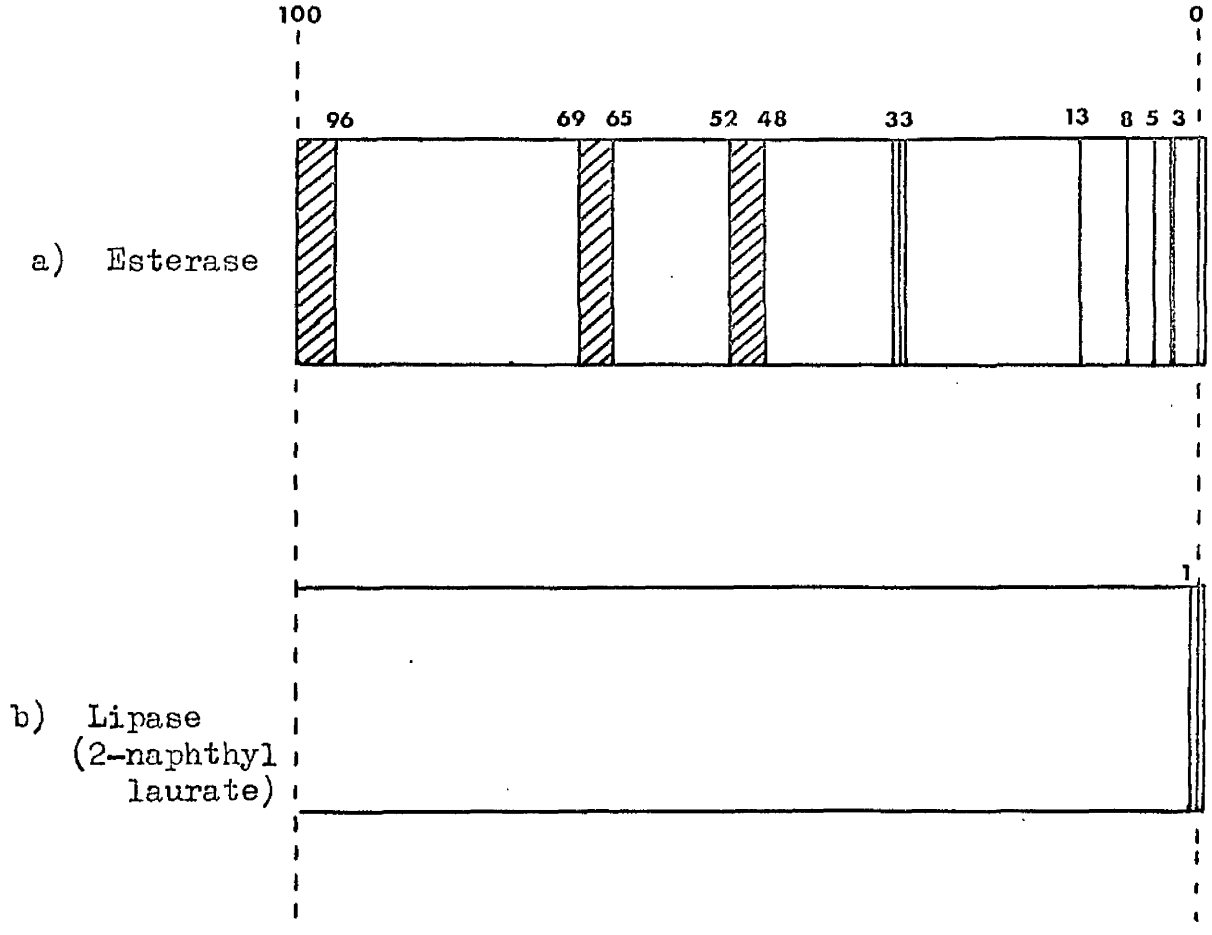


laurate or Tween 80, and precipitation with Tween 40 and Tween 60 occurred at the origin only. However, in the case of Tween 20, the precipitation was marked after 3 days incubation at 30°C, the precipitation occurring to some degree continuously from the origin to 73 mm from the origin, with three intensely precipitating bands that corresponded with the three broad protein bands (Fig. 7e). This reaction was similar to that reported by Sarda, Marchis-Mouren, Constantin and Desnuelle (1957) in their study of pancreatic lipase by paper electrophoresis in which they found that when a number of protein bands were obtained, each band invariably possessed a little lipase activity. This they considered to be probably due to a carry over of lipase by the other proteins forming associations with it.

Thus the use of Tween 20 for demonstrating the Tween-hydrolysing enzyme after electrophoresis was confirmed as being effective, although it was doubtful whether the substrates 2-naphthyl laurate or olive oil would yield positive results.

The esterase pattern of bovine serum (Oxoid Sterile reagent) obtained by electrophoresis on polyacrylamide gel with a discontinuous buffer system is shown in Figure 8 (a). This pattern is significantly different from that obtained by Paul and Fottrell (1961) who reported two esterases in bovine serum using starch gel electrophoresis with a discontinuous buffer system. Similar discrepancies have occurred between the zymograms of other sera obtained by different workers - for example, Paul and Fottrell (1961) distinguished five esterase bands in mouse serum whereas Hunter and Strachan (1961) found nine (and sometimes ten) esterase

(Polyacrylamide gel / discontinuous buffer system)



bands in mouse serum.

Supernatant liquids from centrifuged Tween 80 broth cultures of C.bovis strains 119, 124 and 147 were examined by starch gel electrophoresis using a discontinuous buffer system. In addition the supernatant liquids were also examined after concentration using the Membranfilter ultrafiltration apparatus. After electrophoresis, gel slices were stained with nigrosin to detect proteins, and overlaid with Tween 80 agar and incubated to detect Tween-hydrolysing enzymes. No reactions were obtained. Bacterial cell extracts obtained by the method of Kohn and Reis (1963) were also examined, with negative results - no protein bands being visible and no precipitation occurring with Tween 80.

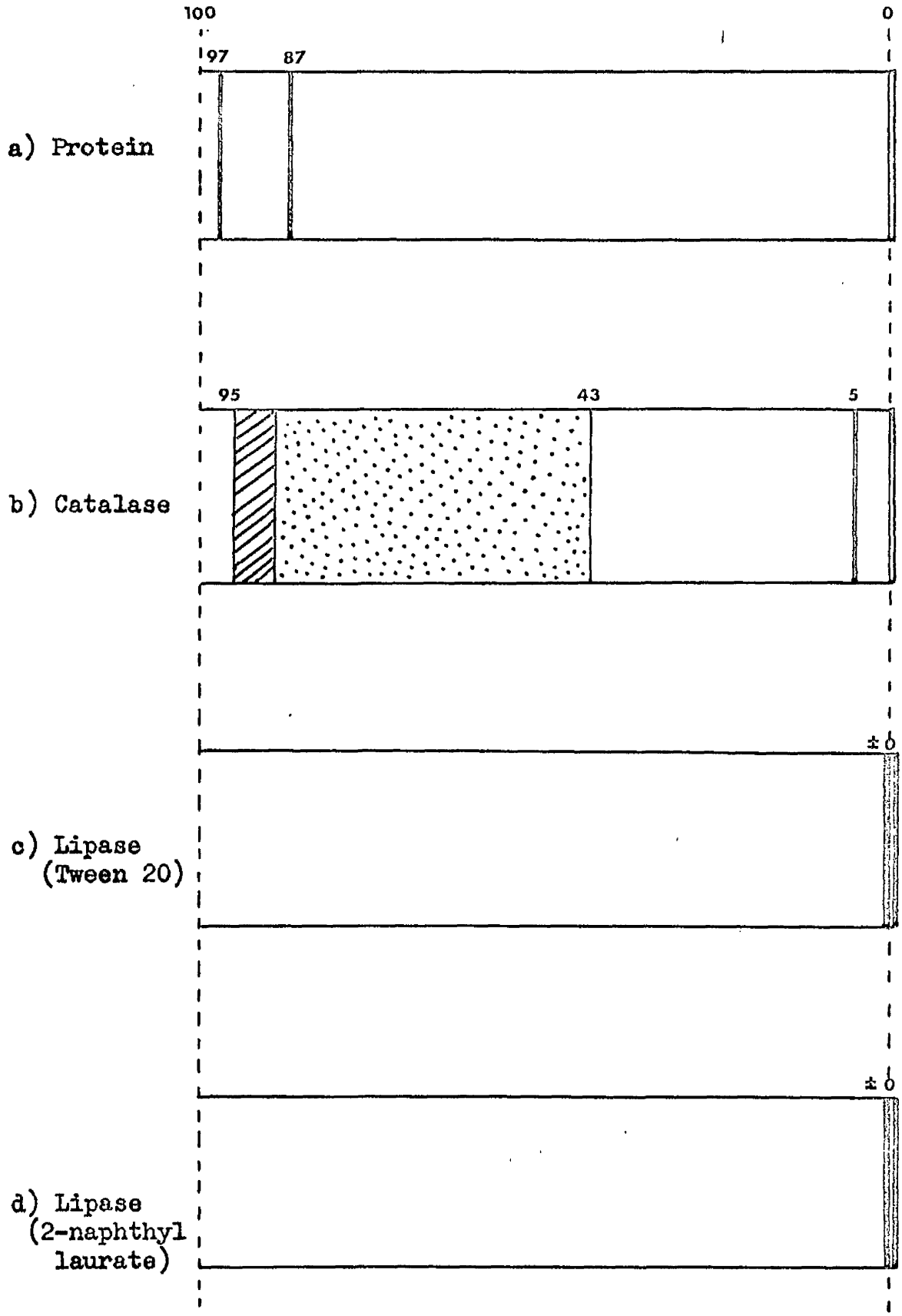
Bacterial cells harvested from 5 per cent serum agar were ground with alumina (McIlwain, 1948) and the extracts obtained were subjected to electrophoresis on starch gel using barbital buffer (pH 7.55). After electrophoresis, the gel slices were examined for the presence of protein by using nigrosin, esterase by using 1-naphthyl acetate and "lipases" by using 2-naphthyl laurate, Tween 20 and Tween 80. There was no evidence of protein bands, esterase or "lipase".

It was found that the protein and esterase patterns of the serum standard obtained by polyacrylamide gel electrophoresis were sharper than those obtained by starch gel electrophoresis. Consequently all subsequent investigations of bacterial extracts were carried out on polyacrylamide gel.

No protein bands were obtained from the supernatant liquids of broth cultures using a polyacrylamide gel/discontinuous buffer system. The absence of protein bands in the supernatant liquids from broth cultures suggested that extracellular enzymes were not produced. Consequently, Corynebacterium bovis strain 124 was grown on serum agar in 1 litre Roux culture flasks to obtain large amounts of bacterial growth and various methods of extracting or disrupting the cells were used, the cell extracts being examined by electrophoresis on polyacrylamide gel with a discontinuous buffer system. It was discovered that the method of Kohn and Reis for extracting bacterial cells gave only one or two very faint and indistinct protein bands. Grinding with alumina resulted in two narrow, rapidly-moving protein bands (Fig. 9a). Acetone extraction of the cells also released protein which was separated by electrophoresis into two narrow, rapidly-moving bands (Fig. 10a), one of which corresponded to a protein band obtained by grinding. The other band occurred at a slightly different position, running at the same speed as the buffer boundary. No esterase reaction was obtained with the ground extract, and the esterase reaction that was obtained with the acetone extract occurred at the origin only (Fig. 10b). A reaction at the origin was obtained using 2-naphthyl laurate in the case of the ground extract (Fig. 9d), but no reaction with 2-naphthyl laurate was obtained in the case of the acetone extract. The alumina-ground extract produced precipitation at the origin with Tween 20 (Fig. 9c), but no reactions were produced with

OF ALUMINA-GROUND EXTRACTS OF C.BOVIS STRAIN 124

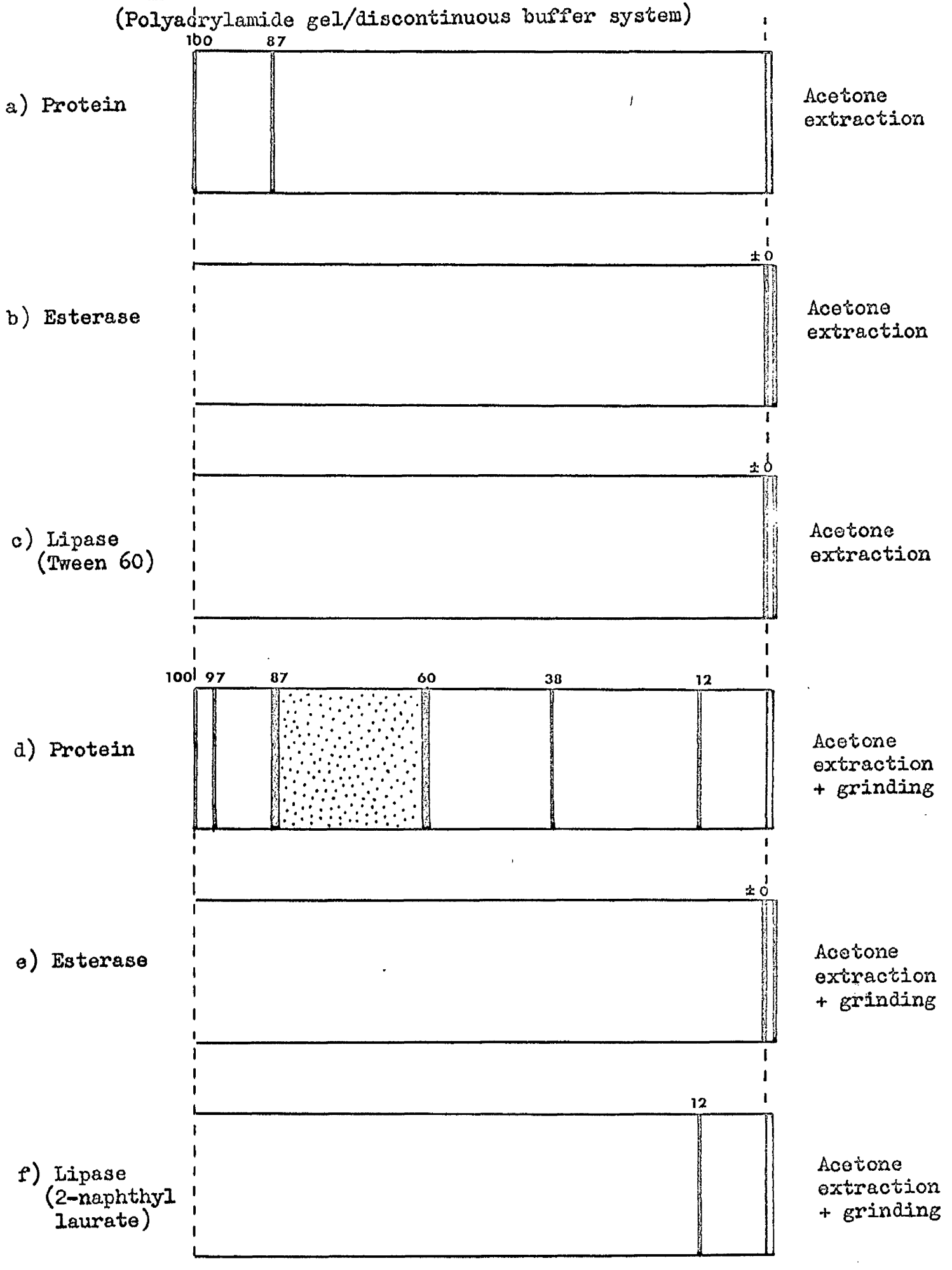
(Polyacrylamide gel/discontinuous buffer system)



No reactions were obtained for esterase, or with the other Tweens.

OF ACETONE EXTRACTS OF C.BOVIS STRAIN 124.

(Polyacrylamide gel/discontinuous buffer system)



No reactions were obtained for catalase, or with the other Tweens.

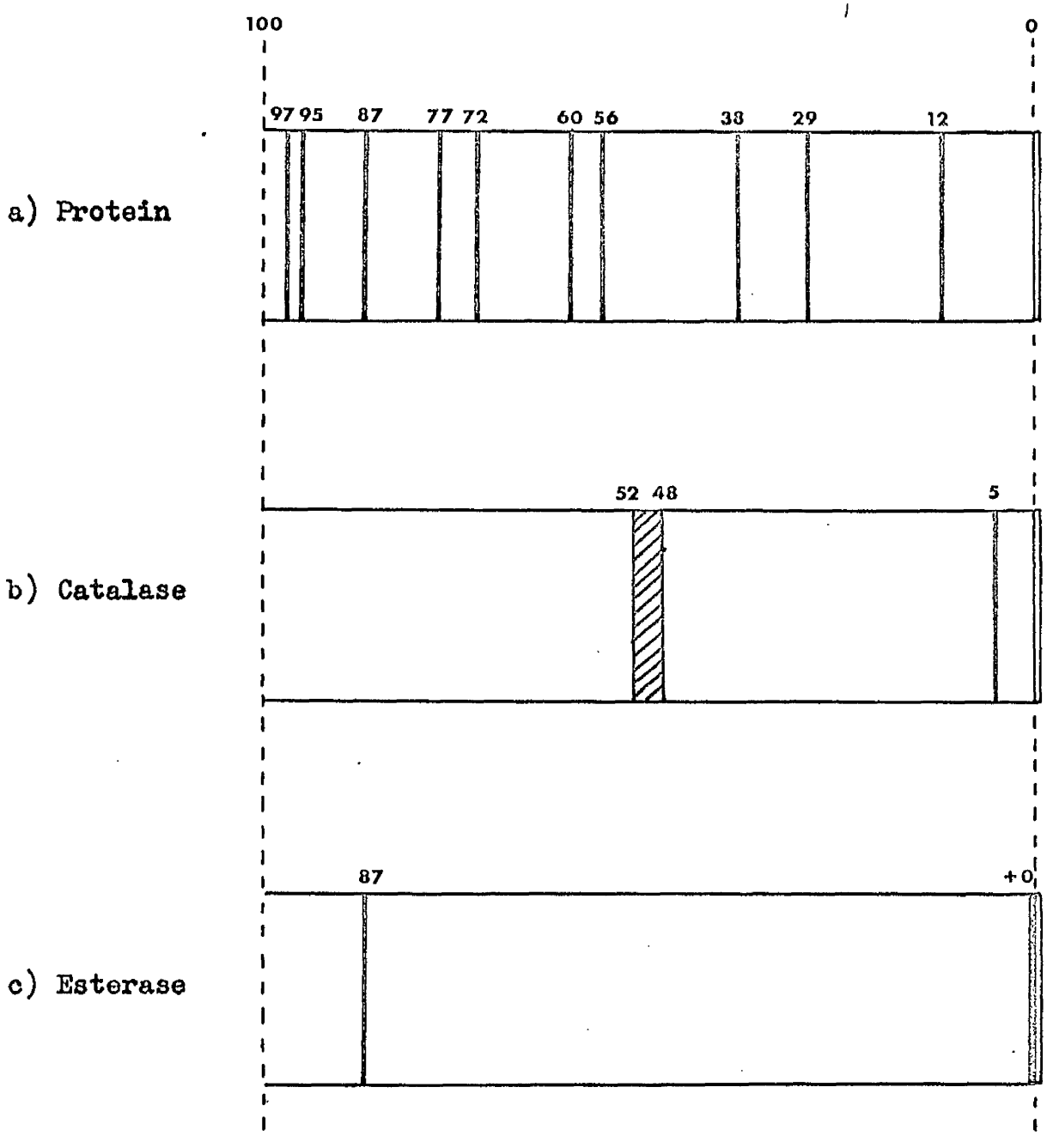
the other Tweens. Tween 20 elicited no reaction with the acetone extract, but there was precipitation at the origin with Tween 60 (Fig.10c). Neither extract produced a reaction with olive oil.

Acetone extraction followed by grinding with alumina proved much more efficient with six deeply stained protein bands being distinguished after electrophoresis (Fig. 10d). There was also an area of lighter staining occurring between two adjacent deeply stained bands. The three most rapidly-moving bands corresponded with the two bands obtained from the ground extract and the very fast-moving band obtained from the acetone extract. An esterase reaction occurred at the origin only. No reaction was obtained with Tweens or olive oil as substrates. However, with 2-naphthyl laurate as the substrate a reaction was obtained at 12 mm from the origin, a reaction that corresponded with the slowest moving protein band.

When ultrasonic disintegration was used to extract the bacterial cells, ten narrow but deeply-stained protein bands were obtained (see Fig.11a), five of which corresponded with protein bands obtained with the alumina-ground acetone extract. Esterase bands occurred at 87 mm and on the leading edge of the sample slit (Fig. 11c). The esterase band at 87 mm corresponded with a protein band which was found in the ground extract, the acetone extract and the alumina-ground acetone extract but in none of these other extracts was esterase activity discernible at this point. No reactions were obtained with the Tweens, olive oil or with 2-naphthyl laurate.

DISINTEGRATES OF C.BOVIS STRAIN 124

(Polyacrylamide gel/discontinuous buffer system)



No reactions were obtained with the Tweens or 2-naphthyl laurate.

Catalase reactions were obtained with two extraction techniques - after grinding with alumina (Fig. 9b), and after ultrasonic disintegration (Fig. 11b). In both cases 2 bands were obtained but only one band (a narrow one at 5 mm) was common to both patterns.

No reactions were obtained with extracts prepared using the Braun disintegrator.

Discussion and conclusions

The long-established use of naphthyl acetate as a substrate for esterase and of naphthyl laurate as a substrate for lipase in colorimetric determinations was based on the earlier definitions (e.g., Gomori, 1952) of these two enzymes, the characteristics of which are summarized in the following table.

TABLE 30. CHARACTERISTICS OF LIPASE AND ESTERASE

	Lipase	Aliesterase
1 Substrate preferences:		
a) chain length of fatty acid	Long (>12)	Short (<12)
b) branching of fatty acid chain	Straight chain	iso chain
c) aliphatic or aromatic nature of fatty acid	Aliphatic	Aromatic
d) Nature of alcohol moiety	Glycerol	Monohydric alcohol
e) Rates of hydrolysis of nitrophenol esters of C_2-C_5 fatty acids	2<3<4<5	2<3>4>5
2 Activators and inhibitors:		
a) Quinine	Inhibition	No effect
b) Arsanilic acid	No effect	Inhibition
c) Fluoride	Slight inhibition	Marked inhibition
d) Bile acids	Activation	Inhibition

As pointed out in Section D the current definition (Recommendations, 1965) of a lipase is that it acts on water-insoluble esters or fats at the ester/water interface in emulsions, whereas an esterase acts on water-soluble substrates. Thus, ideally, a substrate for lipase should be totally insoluble, and a substrate for an aliesterase totally soluble, in water. In practice some substrates used for detecting lipase activity may be slightly soluble in water in which case an esterase could act on the substrate

in the aqueous phase the substrate being continuously replaced from the non-aqueous phase.

On the other hand lipases may act on water-soluble substrates such as triacetin and methyl butyrate if the substrate solution is over-saturated, in which case the substrate may form an emulsion and become accessible to lipase (Desnuelle and Savary, 1963).

Thus, if the present definitions of lipase and esterase are used, there would be justification for the use of naphthyl laurate as a substrate for lipase and naphthyl acetate as a substrate for esterase, only if naphthyl acetate was relatively water-soluble, and naphthyl laurate relatively water-insoluble. There is no reliable evidence concerning the exact solubilities of these two compounds in water, but both are regarded as being water-insoluble and any slightly greater solubility of naphthyl acetate can only be predicted on theoretical grounds. Nevertheless both substrates are used in circumstances in which they exist as oversaturated aqueous solutions (although their solubilities are extremely low), and consequently could be acted on by both esterases and lipases.

In the presence of emulsions lipase molecules probably become activated due to adsorption on to the substrate at the substrate/water interface. As pointed out in Section D it is an open question as to whether the physico-chemical structure of the solution of Tween in water would allow the activation of, and subsequent hydrolysis by, a lipase.

The object of the colorimetric and electrophoretic studies in

this work was to examine the enzymes responsible for the action of C.bovis on Tween, butter-fat and margarine. It was hoped that zone electrophoresis could reveal whether the enzymes responsible for these reactions were identical, and whether any activity against naphthyl laurate determined by colorimetry was due to the enzyme (or enzymes) also responsible for the hydrolysis of Tween or natural substrates.

The results obtained in the first experiment using colorimetry (Table 27) revealed that the broths in which C.bovis had been grown were capable of hydrolysing 2-naphthyl laurate, C.bovis strain 124 demonstrating the greatest activity of the four strains tested. A second, more detailed experiment with C.bovis strain 124 (Tables 28 and 29) in which both cell extracts and cell-free broths were examined showed that the activity of the supernatant liquid from the Tween 80 broth culture was at a maximum after 115 hours incubation, but the high levels obtained in the first experiment were not repeated, and in the second experiment no significant activity (except perhaps a slight taurocholate-activated reaction after incubation for 68 hours) was obtained with the supernatant liquid from the Tween 20 broth culture. In both experiments the hydrolysis of naphthyl laurate by the supernatant liquid was activated by taurocholate, although it was not apparently inhibited by quinine. Activation of lipase by taurocholate is well documented in the case of pancreatic lipase, but taurocholate-activation of microbial lipase has also been described (Patel, Goldberg and Blenden, 1964).

The cell extracts reached a maximum in their hydrolysis of naphthyl laurate after 68-163 hours incubation of the cultures, once again with noticeable activation by taurocholate. The "esterase" levels of the cell extracts (determined with naphthyl acetate) were fairly constant when the organism was grown in Tween 20 broth or Tween 80 broth, but extracts of cells grown in lecithin broth showed a rise in activity against naphthyl acetate with increase in the time of incubation of the culture.

Electrophoresis of C.bovis strain 124 on polyacrylamide gel with a discontinuous buffer system showed that hydrolysis of 2-naphthyl laurate was associated with a protein band at 12 mm when the cells were extracted by grinding an acetone extract with alumina. The only naphthyl acetate- and Tween-hydrolysing activity to be obtained was restricted to the origin, no migration of the enzymes responsible having occurred (Fig. 10). This was the only evidence to be obtained concerning the possible identity of the Tween-hydrolysing enzyme with the enzyme responsible for hydrolysis of naphthyl laurate and/or naphthyl acetate. Ultrasonic disintegration of the cells resulted in an esterase band at 87 mm and another at the leading edge of the sample slit. The 87 mm esterase band corresponded with a protein band. The protein band at 12 mm obtained after ultrasonic disintegration displayed no enzymic activity against naphthyl laurate or any other substrate. The results obtained by electrophoresis indicated that ultrasonic disintegration of the cells was efficient in release of proteins. Ten protein bands were

obtained from such an extract. This corresponded with the protein pattern of C.bovis (NCTC 3224) obtained by Robinson (1966). The catalase pattern was also similar to that detected by Robinson, although the two catalases were more widely separated by the electrophoresis in the present investigation. However, the detection of esterases in the ultrasonic disintegrate was not in accord with the work of Robinson, who was unable to discern esterase activity in C.bovis. The procedure used for the preparation of ultrasonic disintegrates appeared to be satisfactory in that it released, but did not inactivate, the catalases and esterase (naphthyl acetate-hydrolysing enzyme), but it is possible that lipaseswere inactivated by this treatment since the position of the naphthyl laurate-hydrolysing enzyme detectable in the alumina-ground acetone extract at 12 mm was occupied by a protein band in the electrophoretogram of the ultrasonic disintegrate which displayed no reaction with naphthyl laurate. No activity against Tweens was observed after electrophoresis in any position other than at the origin.

From the electrophoresis results it appeared that the Tween-hydrolysing enzyme was not identical with either the naphthyl laurate-hydrolysing enzyme (lipase?) or the naphthyl acetate - hydrolysing enzyme (esterase?). No reaction was obtained when stabilized olive-oil emulsion was used as the substrate. The use of natural substrates such as olive oil or butter for detecting lipase bands in the electrophoretograms is made difficult by their inability to diffuse into the gel, which means that a noticeable reaction probably will be obtained only if the lipase diffuses out of the gel. This did not

occur in this investigation, and the characterization of lipases by zone electrophoresis using natural emulsified substrates can probably only be accomplished if a preparative technique is used, with the elution or extraction of the lipase so that its reactions can be studied in liquid, diphasic systems. Such a technique has been used by Sarda and co-workers (Sarda et al., 1957; Sarda and Desnuelle, 1958; Marchis-Mouren, Sarda and Desnuelle, 1959) in the purification of pancreatic lipase by aqueous extraction, followed by fractional precipitation with ammonium sulphate and acetone, and preparative zone electrophoresis in starch columns.

GENERAL CONCLUSIONS

Corynebacterium bovis should be regarded as a potential pathogen in the udder since it was isolated as the sole organism from 91 of the 389 quarters exhibiting subclinical mastitis and from 13 of the 52 quarters with clinical mastitis.

Of 142 diphtheroids isolated from samples of freshly drawn bovine milk over a period of two years, 127 corresponded morphologically and culturally to Corynebacterium bovis. Five of the remaining strains closely resembled Microbacterium in many characteristics including their heat resistance, being able to survive 15 minutes at 72°C. The main difference between these Microbacterium isolates and the published descriptions of Microbacterium was that the isolates grew better at 37°C than at 30°C. No organisms were isolated that corresponded with C.ulcerans.

During the primary isolation of the organisms from the milk samples, there was no difference in the numbers or size of the colonies obtained on serum agar and blood agar (the medium routinely used for the isolation of mastitis-causing bacteria), but subsequent growth of the isolates was substantially better on serum agar than that obtained on blood agar. It is suggested that this phenomenon may be due to the blood agar being inhibitory toward C.bovis, which is protected from this inhibitory effect during primary isolation by the milk in which it is streaked onto the surface of the blood agar.

The cultural requirement of C.bovis for media containing serum first noted by Evans (1916) and Bendixen (1933), was confirmed, and the cultural requirement for serum could be satisfied by replacement of the

serum by egg-yolk, butter-fat or Tween 80. These compounds acted by satisfying a nutritional requirement of C.bovis rather than by rendering the media such as tryptose lemco agar non-inhibitory by binding traces of free fatty acids, since the isolates of C.bovis were unable to grow in media containing albumin or activated charcoal (both substances known to be capable of rendering free fatty acids non-toxic).

In view of the nutritional requirement of C.bovis, which was satisfied by serum, egg-yolk, butter-fat or Tween 80, it seems likely that the many reports of C.bovis being biochemically inactive were due to the use of basal media incapable of supporting the growth of the organism. When suitable basal media were used, C.bovis was found to be capable of producing acid from a wide range of carbohydrates, of hydrolysing urea and of hydrolysing Tween 80 (and Tween 20). The weak hydrolysis of tributyrin observed by Black (1941) can be enhanced by the use of very heavy inocula, and incubation for 3 days at 37°C followed by 5 days at 20°C. Evidence of lipolysis by C.bovis was obtained on Victoria blue margarine agar, Victoria blue butter-fat agar, and butter-fat agar flooded with saturated copper sulphate solution after incubation, but strains varied in their ability to produce a noticeable reaction. In the case of butter-fat agar in particular, increasing ability to produce a colour reaction when treated with copper sulphate appears to be correlated with a decrease in yellow pigmentation - the deeply yellow-pigmented strains producing no colour reaction with copper sulphate, and white-pigmented strains producing the deepest colour reaction with copper sulphate. It is

interesting to note that this variation in pigmentation only occurred on butter-fat agar. On all other media, the growths of all the isolates of C.bovis were similarly pigmented although the nature of the medium was able to affect the colour of the growth - on serum agar the colonies of C.bovis were invariably greyish-white and although opaque gave the impression of translucency, whereas on egg-yolk agar the colonies were invariably cream-coloured and appeared more opaque. The dark brown discoloration of serum agar noted by Evans (1916) and Bendixen (1933) seemed to be inconstantly produced.

It is proposed that the descriptions of C.bovis, for example such as that given in Bergey's Manual (Breed et al., 1957), be amended to give recognition to the following positive biochemical characteristics: acid is produced from fructose, glucose and glycerol and also from arabinose, xylose, rhamnose, mannitol and sorbitol; urea is hydrolysed; Tween 80 is hydrolysed; lipolysis may occur on butter-fat agar or Victoria blue butter-fat agar; growth is absent or sparse on nutrient agar or tryptose lemco agar, but good growth is obtained on serum agar, egg-yolk agar, butter-fat agar and Tween 80 agar.

By the use of chemically-defined media the cultural requirement of C.bovis for serum, butter-fat, egg-yolk or Tween 80 was shown to be due to a nutritional requirement for oleic acid which, in the defined medium and incubation conditions employed, was required at a concentration of 10 mg per litre of medium, growth only occurring if the oleic acid were rendered non-toxic by the inclusion in the medium of a compound such as albumin or Tween 40. Tween 80 included in the fully defined medium at a concentration of 0.05% (v/v) satisfied the fatty acid

requirement of the organism without the inhibitory effect obtained when free oleic acid was used. Synthetic βγ -dipalmitoyl phosphatidylcholine at a concentration of 80 mg per litre of fully defined medium supported moderate growth of C.bovis but only when solubilized by the addition to the medium of 0.005% Tween 80 (a concentration of Tween 80 which would support only slight growth in the absence of the lecithin). Of the two strains of C.bovis whose vitamin requirements were studied, one had an absolute requirement for both riboflavin and thiamin with growth being stimulated by β-alanine, pyridoxine and biotin. The other strain had complex requirements probably with a number of growth factors being capable of reciprocal replacement. The oleic acid requirement of C.bovis was not capable of being replaced by biotin, but biotin was stimulatory to growth when included in the fully defined medium containing 0.05% Tween 80.

The results obtained using electrophoretic techniques did not result in any conclusive evidence of the identity of, and relationships between, the enzymes responsible for the hydrolysis of Tweens, natural fats and naphthyl esters. The protein and catalase electrophoretograms of ultrasonic disintegrates of C.bovis were similar to those described by Robinson (1966) but in addition a naphthyl acetate-hydrolysing (esterase?) band and a naphthyl laurate-hydrolysing band were also detected.

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