

PATENT SPECIFICATION

782.125



Date of Application and filing Complete Specification Dec. 3, 1954.

No. 35132/54.

Application made in United States of America on Dec. 4, 1953.

Complete Specification Published Sept. 4, 1957.

Index at acceptance :—Class 2(3), AA(1B: 2A3).

International Classification :—C12d.

COMPLETE SPECIFICATION

Production of the Antibiotic Tetracycline by Fermentation

We, BRISTOL LABORATORIES LIMITED, formerly The Angier Chemical Company Limited, a British company, of 211, Blackfriars Road, London, S.E.1, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

10 This invention is concerned with the production of tetracycline by fermentation and more particularly relates to the production of tetracycline by submerged, aerobic fermentation of a chlorotetracycline-producing species of streptomycetes in the presence of an inhibitor of fermentative chlorination.

During the past few years a number of metabolic products of the growth of bacteria and fungi have been isolated and found to possess valuable therapeutic properties. Chlorotetracycline, oxytetracycline and tetracycline have been particularly useful because of their broad spectrum of activity. Of these three, tetracycline is particularly useful because it gives better blood levels and fewer side-reactions and is more stable in alkaline media.

The preferred method of production of tetracycline is by aerobic submerged fermentation but isolation and purification of the product is rendered difficult and expensive in this method by the simultaneous production by the organism of chlorotetracycline as well as tetracycline. Indeed, the former often predominates.

It is an object of the present invention to provide an improved process for the production of tetracycline by fermentation.

It is a further object of the invention to provide a fermentation in which the yield of tetracycline is increased at the expense of that chlorotetracycline which is normally produced.

It is a still further object of the invention to provide a method of fermenting chlorotetracycline-producing organisms in the presence of inhibitors of fermentative chlorination.

tion to produce broths containing tetracycline and substantially free of chlorotetracycline.

We have now discovered a process for the production of tetracycline which comprises growing a culture of a chlorotetracycline-producing species of organism in the presence of an inhibitor of fermentative chlorination until substantial antibacterial activity is imparted to the solution.

According to the present invention there is provided a process for the production of tetracycline which comprises growing under aerobic conditions a culture of a chlorotetracycline-producing species of Streptomyces in an aqueous, nitrogenous nutrient, chloride ion containing, carbohydrate solution under submerged aerobic conditions in the presence of an inhibitor of fermentative chlorination until substantial antibacterial activity is imparted to the solution.

In media not containing chloride ions the chloro-tetracycline-producing species of micro-organism cannot produce the chlorine-containing antibiotic and usually produce tetracycline. According to this invention the production of chlorotetracycline in chloride ion-containing media is prevented by the presence of substances inhibiting the fermentative chlorination.

The inhibitor of fermentative chlorination comprises a bromide, iodide or thiocyanate.

A preferred embodiment of the present invention provides a process for the production of tetracycline which comprises growing under aerobic conditions a culture of a chlorotetracycline-producing species of Streptomyces in an aqueous, nitrogenous nutrient containing, carbohydrate solution under submerged aerobic conditions in the presence of a bromide until substantial antibacterial activity is imparted to the solution. The bromide is preferably present in an amount of from 0.01 to 5.0 per cent by weight of the media.

We have prepared tetracycline by the cultivation under particular controlled conditions of many species of Streptomyces, including

[Price 3s. 6d.]

Chlorina-

21

S. aureofaciens (NRRL 2209) and *Streptomyces viridifaciens* (Number ATCC—11989) which was isolated from a sample of soil. *S. Viridifaciens* (ATCC 11989) is described in copending Application No. 30108/54 (Serial No. 770,065) and elsewhere in this application as BL 567201 (e.g. page 6, line 6; page 7 line 1).

The following is the diffusion plate assay method for determining the activity of tetracycline.

CULTURE MEDIUM

Streptomycin Assay Agar (with yeast extract) was used. A suitable preparation may be made by suspending in one litre of distilled water to a final pH of 6.2 a mixture of 1.5 grams beef extract, 3 grams yeast extract, 6.0 grams peptone (e.g. Gelysate) and 15 grams agar. The suspension is allowed to stand for five minutes, mixed until a uniform suspension is obtained and heated gently with stirring. The suspension is boiled for one or two minutes or until solution has occurred. The culture medium is then dispensed and sterilized at 121° C. (fifteen pounds per square inch of steam pressure, gauge, for fifteen minutes).

INOCULUM

The test organism is *Bacillus subtilis* A.T.C.G. 6633. A spore suspension containing 50,000,000 viable spores per ml. is added to melted assay agar (cooled to 53° C.) to give a final inoculum of 2%.

PREPARATION OF PLATES

Twenty-one ml. of sterile assay agar are placed in level sterile Petri plates and allowed to solidify. Four ml. of inoculated agar are

then distributed evenly over the surface of the base layer. Stainless steel templates containing a series of holes are placed on the medium after the latter has cooled to room temperature and samples are placed within the holes.

BUFFER

A citrate buffer at pH 6.2 is used for making dilutions. This is prepared by mixing 192.12 g. of anhydrous citric acid with 106.3 grams sodium hydroxide in one litre of distilled water and diluting the mixture to one-tenth concentration with distilled water. The pH of the buffer must be checked potentiometrically, and, if necessary, adjusted to pH 6.2 by the addition of citric acid or sodium hydroxide. Variations in pH or concentration of the buffer affect the sizes of inhibition zones markedly. It has not been found necessary to sterilize the buffer. The stock solution is preserved with chloroform or toluene and fresh working solutions prepared daily.

ASSAY

Unknown samples are diluted, if required, in the pH 6.2 citrate buffer. Three holes on each plate are used to receive a single dilution of the sample. Following incubation at 32° C. the diameters of the zones are measured and averaged.

The Streptomycete producing BL 567201 was differentiated from a strain of *S. aureofaciens* (NRRL 2209) obtained from the Northern Regional Research Laboratory, Peoria, Illinois where it had been deposited as an authentic aureomycin-producing strain, by observation of growth characteristic on glycerol asparagine beef extract agar and Czapek-Dox agar containing 1% dextrin.

GLYCEROL ASPARAGINE BEEF EXTRACT AGAR

Glycerol	-	1%
Asparagine	-	0.05%
Beef extract	-	0.2%
K ₂ HPO ₄	-	0.05%
Agar	-	1.5%

pH 7.2

	BL 567201	<i>Streptomyces aureofaciens</i>
Growth:	Good	Good
Sporulation:	Good	Good
Diffusible pigment:	Yellowish-green	None
Spinal formation:	Abundant, loosely wound	None
Aerial hyphae:	Mouse-grey	Rose-grey
Reverse:	Brown	Olive-drab

DEXTRIN CZAPEK-DOX

NaNO ₃	-	-	0.2%
K ₂ HPO ₄	-	-	0.1%
MgSO ₄	-	-	0.05%
KCl	-	-	0.05%
FeSO ₄	-	-	trace
Agar	-	-	1.5%

pH 7.2

	BL 567201	<i>Streptomyces aureofaciens</i>
Growth:	Fair to good	Fair
Sporulation:	Good	Poor
Diffusible pigment:	None	None
Spiral formation:	Abundant, loosely-wound	Sparse, very loosely-wound
Aerial hyphae:	Mouse-grey	Buff to gray
Reverse:	Light-brown	Buff to tan

- 10 The *Streptomyces* BL 567201 is further characterised by production of an intense bluish-green pigment when grown in submerged culture in a medium containing 1% sucrose, 1% soy bean meal, 1% soy peptone, 1.5% KH_2PO_4 , and 0.5% $(\text{NH}_4)_2\text{HPO}_4$.
- 15 *Streptomyces aureofaciens* (NRRL 2209) does not produce this pigment.

- This invention embraces a process for growing chlorotetracycline-producing species of *Streptomyces* at about 24°—30° C. under submerged conditions of agitation and aeration on media, consisting of a source of carbon, a source of nitrogen, a source of growth substances, mineral salts such as potassium phosphate, magnesium sulphate, and sodium nitrate, and when desired, a buffering agent such as calcium carbonate, with the addition to the media of a metabolic inhibitor of fermentative chlorination. Such metabolic inhibitors of chlorination include bromide, iodide, and thiocyanate ions. These are added to the media in the form of their compounds, such as alkali metal or alkaline earth metal salts. The preferred inhibitor is bromide ion; it is conveniently added as sodium bromide, potassium bromide, calcium bromide, ammonium bromide or hydrobromic acid. In terms of sodium bromide, useful amounts lie in the range of 0.01 to 5.0 percent by weight of the media; about 0.1 to 2.0 percent is preferred.
- 40 It is theorized, but not certain, that the inhibition of fermentative chlorination demonstrated by this invention may be produced by interference with enzymatic processes and perhaps by the process commonly referred to as "competitive inhibition". However, this invention is not dependent upon, nor restricted to, any such theory.

- As a source of carbon in the nutrient medium one of the following is used:—
- 50 Ordinary starch Xylose
Soluble starch Arabinose
Sucrose Rhamnose
Glucose Fructose
55 Maltose Lactose
Dextrose Inulin
Glycerol Dextrins
Galactose

- These carbon sources are supplied to the medium in purified form or in the form of concentrates. The amount of such carbon sources for best antibiotic production in the

medium varies considerably, from about 0.5% to 5% by weight of the total weight of the fermentation medium. 65

Suitable sources of nitrogen, including some sources of growth substances, for the fermentation process include a wide variety of substances such as:—

- Amino acids 70
Casein, both hydrolysed and unhydrolysed
Fish meal
Soy bean meal
Meat extracts
Liver cake 75
Urea
Nitrates
Ammonium compounds
Distillers grain slop
Corn-steeping liquor 80
~~Wheat-steeping liquor~~ *Wheat-*
Whey or whey concentrates
Acid hydrolysed corn gluten
Acid hydrolysed wheat gluten
Peptone 85
Offals
Brewers yeast
Cottonseed meal
Lactalbumin
Tryptone 90

These proteinaceous ingredients need not be applied in a high degree of purity; the less pure materials which carry traces of growth factors and considerable quantities of mineral nutrients are suitable for use. It is not possible, of course, because of the crude nature of many of these nitrogenous substances to specify definite proportions of the material to be added. An amount of about 0.1% to 5.0% by weight on a solid basis describes the useful range of nitrogenous substances to be added to the media in most cases. 100

The pH of the fermentation medium should be 6.0 to 6.2 at the start of the fermentation. The preferred temperature of the fermentation process is about 26° to 28° C. The maximum yield of product is usually obtained within 1 to 3 days, varying with the method of cultivating the *Streptomyces*. 105

Following is a description by way of example of methods of carrying the invention into effect:— 110

EXAMPLE I

For the production of tetracycline, an inoculum is prepared in a fermentation medium containing, by weight, 115

- 1% Corn steep
1% Sucrose
0.5% $(\text{NH}_4)_2\text{HPO}_4$
1.5% KH_2PO_4
5 0.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
0.5% NaBr
pH 6.2 to 6.4
made up in a volume of 2500 ml. and introduced into a $2\frac{1}{2}$ gallon bottle. The medium is
10 sterilized with steam at 118° to 120° for one hour. When cool, the medium is inoculated with about 0.5%, by volume, of a turbid, aqueous spore suspension of the *Streptomyces* from an agar slant. The contents of the bottle
15 are then incubated at 26° to 28° C. for 48 hours on a reciprocating-type shaker and sterile air blown over the surface of the liquid. From the inoculum bottle the broth containing the *Streptomyces* sp. is transferred into
20 the tank fermenter under completely aseptic conditions. When desired, the liquor may be processed as described below and the tetracycline isolated.
- The tetracycline produced by *Streptomyces*
25 BL 567201, *Streptomyces aureofaciens* (NRRL 2209), and other tetracycline and chlorotetracycline producing species of *Streptomyces* may be prepared on a large scale by submerged or deep culture. Stationary vat
30 fermenters equipped with suitable agitation and aeration have been found to be useful for this purpose. A nutrient medium consisting of 56.8 litres of corn steep, 56.8 kg. sucrose, 28.4 kg. $(\text{NH}_4)_2\text{HPO}_4$, 85.2 kg. KH_2PO_4 , 11.3 kg.
35 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 28.4 kg. NaBr, and water to make 1500 gallons is suitable. This medium may be prepared in a 2000 gallon glass-lined fermenter equipped with a water-jacket for temperature control, a suitable stainless steel
40 agitator and a suitable sparging device for aeration. The medium is sterilized by heating with steam under pressure and is then cooled. After sterilization the hydrogen ion concentration of the medium should be approximately pH 6.2. The nutrient medium is inoculated with 15%, by volume, of a vegetative culture grown either in a similar type fermenter previously inoculated with an inoculum described earlier or with an
50 inoculum prepared in the laboratory. The culture in the 2000 gallon fermenter is incubated at a temperature of 83° F. for about 44 hours. During the incubation the impeller is revolved at the rate of 90 R.P.M. and sterile
55 air is introduced into the medium through the sparger at the rate of 100 cubic feet per minute. At the end of the incubation period the culture fluid normally contains a substantial amount of tetracycline which exceeds the
60 amount of chlorotetracycline present. The tetracycline is isolated as described below; the solids obtained before recrystallization contain at least nineteen parts tetracycline for every part of chlorotetracycline.

EXAMPLE II

65 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% Wheat gluten
1% Glycerol
0.05% Distiller's solubles
0.1% CaCO_3
0.5% NaBr

65

70

75

EXAMPLE III

70 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% Cottonseed meal
1% Glucose
0.05% Distiller's solubles
0.1% CaCO_3
0.5% NaBr

85

EXAMPLE IV

90 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% Corn steep liquor
1% "Cerelose"
0.1% CaCO_3
1.0% NaBr

95

EXAMPLE V

100 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% Soy bean meal
1% "Cerelose"
0.05% Yeast extract
0.1% CaCO_3
0.1% NaBr

105

EXAMPLE VI

110 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 3% Soy bean meal
0.5% Corn starch
0.1% N—Z—amine B (Enzymatic digest of casein)
0.3% NaNO_3
0.5% CaCO_3
0.5% KBr

115

120

EXAMPLE VII

125 Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% N-Z-Amine B
 1% "Cerelese"
 0.5% Yeast extract
 0.1% CaCO_3
 5 0.5% CaBr_2
 "Cerelese" is a Registered Trade Mark.

EXAMPLE VIII

- Fermentation liquor containing tetracycline, and no more than one part chlorotetracycline
 10 per two parts tetracycline, is produced by following the procedure of Example I, using the following culture medium.

- 1% Sucrose
 1% Soy bean meal
 15 1% Soy peptone
 1.5% KH_2PO_4
 0.5% $(\text{NH}_4)_2\text{HPO}_4$
 0.5% NaBr

EXAMPLE IX

- 20 The procedure of Example I was carried out both with and without 0.5% sodium bromide under otherwise comparable conditions using *Streptomyces* BL 567201; the amounts of tetracycline and chlorotetracycline
 25 in the resulting broths were determined by paper-strip chromatography or by differential bio-assay on pH 6.2 and 8.0 agar.

- The broth from a 120-gallon tank containing 80 gallons media, including 0.5% sodium
 30 bromide, after 20 and 44 hours fermentation contained at least nineteen parts tetracycline per part of chlorotetracycline. By bio-assay

after 20 hours, the broth contained 85 mcg./ml. tetracycline and 3 mcg./ml. chlorotetracycline. 35

The broth from a 120-gallon tank containing 80 gallons media, containing no added bromide ion, after 44 hours fermentation contained no more than one to two parts tetracycline per eight parts chlorotetracycline. 40

The broth from a 600-gallon tank containing 350 gallons media, containing no added bromide ion, after 45 hours fermentation contained no more than one to two parts tetracycline per eight parts chlorotetracycline. 45

The broth from another 600-gallon tank containing 300 gallons media, containing no added bromide ion, after 30 hours fermentation contained no more than one part tetracycline per nine parts chlorotetracycline. 50

EXAMPLE X

The various media tabulated below were prepared in 200 ml. quantities per one litre Erlenmeyer flask in duplicate, autoclaved 15 minutes at 15 pounds, cooled and inoculated
 55 with a 24 hour vegetative inoculum of *Streptomyces* BL-567201 developed in corn-steep medium at the rate of 1 ml. inoculum/200 ml. medium. The inoculated flasks were agitated on a reciprocating shaker at 28° C. for 48
 60 hours and harvested for assay.

The harvested broths were centrifuged and assayed by paper-strip chromatography.

MEDIA AND ASSAY RESULTS

	Run number				
	1	2	3	4	5
65 Sucrose, % - - -	1	1	1	1	1
KH_2PO_4 , % - - -	1.5	1.5	1.5	1.5	1.5
$(\text{NH}_4)_2\text{HPO}_4$, % - - -	0.5	0.5	0.5	0.5	0.5
70 MgSO_4 , % - - -	0.2	0.2	0.2	0.2	0.2
Corn steep, % - - -	1	1	1	1	1
NaBr , % - - -	0	0.5	0	0.5	1.0
Harvest pH - - -	5.4	5.3	—	—	—
Chlorotetracycline, % - - -	100	10	95	<5	<5
75 Tetracycline, % - - -	0	90	5	>95	>95

Run 2 contained about 100 mcg./ml. of tetracycline.

EXAMPLE XI

- The NaBr of Example X was replaced in
 80 turn by KBr , CaBr_2 , NH_4Br , KI (less than 0.5%) and NaSCN , and the same increased production of tetracycline at the expense of chlorotetracycline was observed.

EXAMPLE XII

- 85 Using *Streptomyces aureofaciens* (NRRL-2209), the addition of bromide, iodide, or thiocyanate ions to the media as in Example

X was found to increase production of tetracycline at the expense of chlorotetracycline, particularly when the media was altered to
 90 suit the strain and to facilitate maximum antibiotic production (e.g. see U.S. Specification 2,482,055).

After fermentation has been completed, tetracycline is recovered from the broth, for
 95 example, by filtering to remove the mycelium, stirring the broth (preferably at about pH 8.5) with butanol or methyl isobutyl ketone, separating the solvent layer containing the tetracycline, concentrating it to small volume 100

by distillation, and mixing it with a petroleum ether e.g., Skellysolve C, to precipitate solid tetracycline as the base, if the extracted broth was at alkaline pH and as the hydrochloride if, for example, the broth had been acidified before extraction with hydrochloric acid. The solid tetracycline so produced may be further purified by slurrying into ammonium hydroxide and also by adsorption from a solution of the free base onto a chromatographic adsorbent (e.g. a silica such as Florisil), followed by washing (e.g. with acetone or methanol) to remove impurities and then elution with an acid. The purified tetracycline acid salt in the eluate is then recovered by e.g. crystallization, precipitation or lyophilization.

Various salts of tetracycline may be prepared, most simply by adding the desired acid, mineral or organic, to the antibiotic in water until a clear solution is obtained. The solid salts may be prepared by adjusting the pH of such a solution of a tetracycline salt to a point just below that at which the antibiotic would begin to separate. The solution may then be dried, as by subjecting the frozen solution to a vacuum. Acid salts of tetracycline are obtained by evaporation of a solution of the salt in water at a low pH. Examples of mineral acids which may be used are hydrochloric acid, sulphuric acid, and phosphoric acid, and examples of organic acids which may be used are citric acid, tartaric acid and gluconic acid. Since tetracycline is amphoteric, salts of various metallic elements with the antibiotic may be prepared; in particular, the alkali metal salts of tetracycline are formed by treating an aqueous suspension of the antibiotic with an alkali metal hydroxide. The solid metal salts of tetracycline are obtained by the evaporation under vacuum of an aqueous solution of the antibiotic at a suitable pH.

The tetracycline produced in the above procedures is conclusively characterised, even when contaminated by similar organic chemicals, by its "spectrum" of activity (i.e. degree of migration or R_f values) using a series of solvents in the procedure known as paper strip chromatography. This technique is a relatively new but already well-established procedure for the identification of organic compounds; as with infra-red maxima the R_f values in a series of solvent systems are a unique and reproducible characteristic of a given chemical and serve as a "fingerprint".

The procedure used is as follows. Strips of ash-free, dense, highly retentive filter paper, one-half inch wide and 58 cm. long, are suspended at constant room temperature in a

protected area (i.e., in a large jar) from the edge of a dish. The top of the strip is in contact with a supply of the solvent system (also called developing phase) in the dish: the bottom of the strip hangs free and does not reach the supply of solvent system (or volatile components thereof) placed below the hanging strips to facilitate saturation of the air with the solvent in use.

The product to be examined (i.e., in a fermentation broth or as an isolated solid) is placed on a marked spot at the top of this portion of the strip which hangs free in the air. In the case of a solid, it is dissolved in a convenient solvent. The amounts used are those giving a convenient zone size on final assay as determined by simple trial. For example, a useful amount is 5 microlitres (0.005 ml.) of a solution containing 1 mgm./ml. solvent of tetracycline for assay vs. *B. subtilis*. The strip is dried and then placed in position in the dish, which contains the selected solvent system. The solvent is allowed to migrate downward, that is, to develop the strip, until the solvent front reaches the bottom of the strip. This requires about fifteen hours; the surrounding atmosphere is maintained at a constant temperature free from drafts and saturated with solvent vapour from a pool below.

The strip is then removed, air-dried, and placed on a tray of agar of controlled pH (here 6.2) inoculated with a test organism, in this case *B. subtilis*. After standing in the refrigerator overnight, the strips are removed, and the trays are marked for identification, incubated overnight and either read directly or photographed to give a permanent record.

The outline of the whole strip is visible. The location of each antibiotic agent on the strip is marked by a clear area, which contrasts with the turbid area where the organism has grown. The strip as shown on the photograph is marked off into zones representing 5, 10, 10, 10, 10, 10, 10, 10, 10, and 15 per cent of the distance from the point of sample application to the bottom, respectively, and these zones are described as having R_f values of 1 to 10 inclusive. A small spot in the exact centre would thus have R_f 6, while a larger spot would extend into the adjacent zones and have R_f 5, 6, 7 as the entire zone is counted.

Using this technique, the R_f spectra of tetracycline is found to be as follows, using 5 microlitres of a 1 mgm./ml. solution as a sample and assaying with *B. subtilis* in pH 6.2 agar, in the twelve solvent systems:—

Solvent system		A	B	C	D	E	F	G	H	I	J	K	L
120	R _f value	6,7	5,6	4,5	4,5,6	1	8,9,10	6,7	8	5	9,10	6,7,8	3,4,5

The composition of these solvent systems is as follows:—

- A—Water
- B—10% aqueous sodium citrate
- C—40% aqueous sodium citrate
- D—Butanol saturated with water
- E—Dry methyl isobutyl ketone
- F—A mixture of 100 parts of 80% methanol and 10.5 parts piperidine adjusted to pH 9.5 with acetic acid.
- G—A mixture by volume of 80 parts methanol, 5 parts glacial acetic acid and 15 parts water.

- H—Butanol saturated with water and containing 2% *p*-toluenesulphonic acid.

I—A mixture of 100 mls. of butanol saturated with water and 5 ml. glacial acetic acid.

- J—Butanol saturated with water and containing 2% *p*-toluenesulphonic acid plus 2% piperidine.

K—100 ml. of saturated butanol in water, and 2% *p*-toluenesulphonic acid plus 2 mls. piperidine plus 2% lauric acid.

- L—A mixture of two parts isoamyl alcohol and one part carbon tetrachloride, saturated with 10% aqueous sodium citrate. In this case, before applying the sample, the strips are saturated with 10% aqueous sodium citrate adjusted to pH 5.7 with citric acid and dried.

The properties of tetracycline are described in the Journal of the American Chemical Society, volume 75, pages 4621—23, 1953.

- A sample of "tetracycline" free base was prepared according to the literature from chlorotetracycline and found to melt at 170°—175° C. and to contain 0.71 percent chlorine, indicating a contamination with about 10 percent unreacted chlorotetracycline. This sample was shown to be identical with a sample of tetracycline produced by fermentation, and all impurities were distinguished, by examination of these samples and samples of chlorotetracycline and oxytetracycline, both alone and as mixtures, by paper strip chromatography (particularly using solvent systems D and L).

- Tetracycline lost about 28% of its activity in 48 hours in pH 8.0 buffer at 37° C. at about 0.7 mgm./ml.; chlorotetracycline lost about 50% of its activity in 14 hours and oxytetracycline lost about 50% of its activity in 26 hours under the same conditions.

- In addition, tetracycline is clearly differentiated from chlorotetracycline and oxytetracycline by assay on pH 8.0 agar, on which chlorotetracycline and oxytetracycline appear much less active than tetracycline, and simultaneous assay on pH 6.2 agar.

- Tetracycline may thus be purified from contaminating amounts of chlorotetracycline or oxytetracycline by maintaining an aqueous solution at about pH 8.0 until all the chlorotetracycline or oxytetracycline is decomposed and then isolating purified tetracycline. Such

an operation is greatly facilitated by a process, such as that of the present invention, which holds the amount of chlorotetracycline present down to a minimum.

The following salts are also useful as inhibitors of fermentative chlorination in the present invention when used in amounts just low enough to be non-toxic: sodium acetate, sodium aluminate, sodium aluminium fluoride, sodium aluminium trisilicate, sodium aluminium sulphate, sodium pyroantimonate, sodium orthoarsenate, sodium azide, sodium benzene sulphonate, sodium benzoate, sodium metaborate, sodium tetraborate, sodium perborate, sodium bromate, sodium bromoaurate, sodium bromoplatinate, sodium cacodylate, sodium *d*-camphorate, sodium chromate, sodium dichromate, sodium cinnamate, sodium citrate, sodium cuprocyanide, sodium cyanate, sodium cyanide, sodium enanthate, sodium ethylsulphate, sodium ferricyanide, sodium ferrite, sodium ferrocyanide, sodium fluoantimonate, sodium fluophosphate, sodium fluoride, sodium fluosilicate, sodium formate, sodium metagermanate, sodium glycerophosphate, sodium iodate, sodium metaperiodate, sodium iron oxalate, sodium isocyanate, sodium lactate, sodium magnesium sulphate, sodium manganate, sodium permanganate, sodium molybdate, sodium hyponitrite, sodium nitrite, sodium nitroprusside, sodium oleate, sodium oxalate, sodium hypophosphate, sodium pyrophosphate, sodium hexametaphosphate, sodium hypophosphate, sodium orthophosphate, sodium phthalate, sodium picrate, sodium metaphosphate, sodium salicylate, sodium selenate, sodium selenite, sodium metasilicate, sodium stannate, sodium stearate, sodium succinate, sodium sulphanilate, sodium pyrosulphate, sodium monosulphide, sodium sulphide, sodium hyposulphite, sodium tellurate, sodium tellurite, sodium thioantimonate, sodium thiocarbonate, sodium dithionate, sodium thiosulphate, sodium xanthate, sodium zinc uranyl acetate, sodium zirconium fluoride, and water soluble metallic salts of these anions, such as potassium, calcium, barium, strontium, zinc, iron and magnesium as well as ammonium and substituted ammonium salts and corresponding free acids.

What we claim is:—

1. A process for the production of tetracycline which comprises growing under aerobic conditions a culture of chlorotetracycline-producing species of Streptomyces in an aqueous, nitrogenous nutrient chloride ion, containing, carbohydrate solution under submerged aerobic conditions in the presence of an inhibitor of fermentative chlorination until substantial antibacterial activity is imparted to the solution.

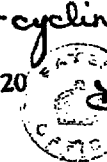
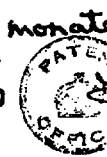
2. A process as claimed in Claim 1 in which the tetracycline produced is recovered from the fermentation broth.

3. A process as claimed in Claim 1 or Claim 130



chlorotetracycline

tetracycline





The composition of these solvent systems is as follows:—

- A—Water
B—10% aqueous sodium citrate
C—40% aqueous sodium citrate
D—Butanol saturated with water
E—Dry methyl isobutyl ketone
F—A mixture of 100 parts of 80% methanol and 10.5 parts piperidine adjusted to
10 pH 9.5 with acetic acid.
G—A mixture by volume of 80 parts methanol, 5 parts glacial acetic acid and 15 parts water.
H—Butanol saturated with water and containing 2% *p*-toluenesulphonic acid.
15 I—A mixture of 100 mls. of butanol saturated with water and 5 ml. glacial acetic acid.
J—Butanol saturated with water and containing 2% *p*-toluenesulphonic acid plus 2%
20 piperidine.
K—100 ml. of saturated butanol in water, and 2% *p*-toluenesulphonic acid plus 2 mls. piperidine plus 2% lauric acid.

- L—A mixture of two parts isoamyl alcohol
25 and one part carbon tetrachloride, saturated with 10% aqueous sodium citrate. In this case, before applying the sample, the strips are saturated with 10% aqueous sodium citrate adjusted to pH 5.7 with citric acid and
30 dried.

The properties of tetracycline are described in the Journal of the American Chemical Society, volume 75, pages 4621—23, 1953.

- A sample of "tetracycline" free base was
35 prepared according to the literature from chlorotetracycline and found to melt at 170°—175° C. and to contain 0.71 percent chlorine, indicating a contamination with about 10 percent unreacted chlorotetracycline. This
40 sample was shown to be identical with a sample of tetracycline produced by fermentation, and all impurities were distinguished, by examination of these samples and samples of chlorotetracycline and oxytetracycline, both
45 alone and as mixtures, by paper strip chromatography (particularly using solvent systems D and L).

- Tetracycline lost about 28% of its activity in 48 hours in pH 8.0 buffer at 37° C. at about
50 0.7 mgm./ml.; chlorotetracycline lost about 50% of its activity in 14 hours and oxytetracycline lost about 50% of its activity in 26 hours under the same conditions.

- In addition, tetracycline is clearly differentiated from chlorotetracycline and oxytetracycline by assay on pH 8.0 agar, on which chlorotetracycline and oxytetracycline appear much less active than tetracycline, and simultaneous assay on pH 6.2 agar.

- 60 Tetracycline may thus be purified from contaminating amounts of chlorotetracycline or oxytetracycline by maintaining an aqueous solution at about pH 8.0 until all the chlorotetracycline or oxytetracycline is decomposed
65 and then isolating purified tetracycline. Such

an operation is greatly facilitated by a process, such as that of the present invention, which holds the amount of chlorotetracycline present down to a minimum.

The following salts are also useful as 70 inhibitors of fermentative chlorination in the present invention when used in amounts just low enough to be non-toxic: sodium acetate, sodium aluminate, sodium aluminium fluoride, sodium aluminium trisilicate, sodium alu- 75 minium sulphate, sodium pyroantimonate, sodium orthoarsenate, sodium azide, sodium benzene sulphonate, sodium benzoate, sodium metaborate, sodium tetraborate, sodium perborate, sodium bromate, sodium bromoaurate, 80 sodium bromoplatinate, sodium cacodylate, sodium *d*-camphorate, sodium chromate, sodium dichromate, sodium cinnamate, sodium citrate, sodium cuprocyanide, sodium cyanate, sodium cyanide, sodium enanthate, sodium 85 ethylsulphate, sodium ferricyanide, sodium ferrite, sodium ferrocyanide, sodium fluoanti-
monate, sodium fluophosphate, sodium fluoride, sodium fluosilicate, sodium formate, sodium metagermanate, sodium glycerophos- 90 phate, sodium iodate, sodium metaperiodate, sodium iron oxalate, sodium isocyanate, sodium lactate, sodium magnesium sulphate, sodium manganate, sodium permanganate, sodium molybdate, sodium hyponitrite, 95 sodium nitrite, sodium nitroprusside, sodium oleate, sodium oxalate, sodium hypophosphate, sodium pyrophosphate, sodium hexameta-
phosphate, sodium hypophosphate, sodium orthophosphate, sodium phthalate, sodium picrate, 100 sodium metaplumbate, sodium salicylate, sodium selenate, sodium selenite, sodium metasilicate, sodium stannate, sodium stearate, sodium succinate, sodium sulphanilate, sodium pyrosulphate, sodium monosulphide, 105 sodium sulphide, sodium hyposulphite, sodium tellurate, sodium tellurite, sodium thioantimonate, sodium thiocarbonate, sodium dithionate, sodium thiosulphate, sodium xanthate, sodium zinc uranyl acetate, sodium zirconium fluoride, and water soluble metallic salts of these anions, such as potassium, calcium, barium, strontium, zinc, iron and magnesium as well as ammonium and substituted ammonium salts and corresponding free acids. 115

What we claim is:—

1. A process for the production of tetracycline which comprises growing under aerobic conditions a culture of chlorotetracycline-producing species of *Streptomyces* in an aqueous, nitrogenous nutrient chloride ion, containing, carbohydrate solution under submerged aerobic conditions in the presence of an inhibitor of fermentative chlorination until substantial antibacterial activity is imparted 120 to the solution.

2. A process as claimed in Claim 1 in which the tetracycline produced is recovered from the fermentation broth.

3. A process as claimed in Claim 1 or Claim 130

chlorotetracycline

tetracycline

2 in which the inhibitor of fermentative chlorination is present in an amount of from 0.01 to 5.0 per cent by weight of the media.

3 4. A process as claimed in any one of the preceding claims in which the inhibitor of fermentative chlorination comprises a bromide, iodide or thiocyanate.

10 5. A process as claimed in any one of the preceding claims in which the inhibitor of fermentative chlorination comprises an alkali metal or alkaline earth metal bromide, iodide, or thiocyanate.

15 6. A process as claimed in any one of Claims 1 to 5 in which the inhibitor of fermentative chlorination comprises sodium bromide

7. A process as claimed in any one of Claims 1 to 5 in which the inhibitor of fermentative chlorination comprises potassium bromide.

20 8. A process as claimed in any one of Claims 1 to 5 in which the inhibitor of fermentative chlorination comprises calcium bromide.

9. A process as claimed in any one of Claims 1 to 4 in which the inhibitor of fermentative chlorination comprises ammonium 25 bromide.

10. A process as claimed in any one of Claims 1 to 4 in which the inhibitor of fermentative chlorination comprises hydrogen 30 bromide.

11. A process for the production of tetracycline substantially as described with reference to any one of the Examples I to XII hereinbefore set forth.

12. Tetracycline when produced by a process 35 as claimed in any one of the preceding Claims 1 to 11.

BOULT, WADE & TENNANT,
111 & 112, Hatton Garden, London, E.C.1,
Chartered Patent Agents.

Reference has been directed in pursuance of Section 9, subsection (1) of the Patents Act, 1949 to Patent No. 747,779.

Leamington Spa: Printed for Her Majesty's Stationery Office, by the Courier Press,—1957.
Published at the Patent Office, 25, Southampton Buildings, London, W.C.2, from which copies may be obtained.



4 NOV 1956