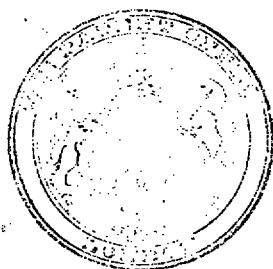


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PATENT SPECIFICATION

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International Classification:—A01k. A61k. C12d.

COMPLETE SPECIFICATION

Production of Tetracycline by Fermentation

We, AMERICAN CYANAMID COMPANY, a corporation organized under the laws of the State of Maine, United States of America, of 30, Rockefeller Plaza, New York, State of New York, United States of America, 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:—

The present invention relates to a new and therapeutically useful broad-spectrum antibiotic substance defined as tetracycline, to its production by fermentation, and to recovery and purification thereof.

In accordance with the present invention there is provided a process of producing an antibiotic substance defined as tetracycline which comprises inoculating and aerobically fermenting an aqueous nutrient medium containing assimilable sources of carbon and nitrogen and mineral substances with a micro-organism of the genus *Streptomyces* which tends to produce chlortetracycline in the presence of chloride ions, said method being characterized by controlling the chloride ion content of the nutrient medium so that the formation of chlortetracycline is restricted and tetracycline is formed as the predominating antibiotic.

We have discovered that under conditions which will be explained hereinafter, micro-organisms of the genus *Streptomyces* produce in a fermentation medium a high concentration of an antibiotic substance different from those heretofore known, and, in particular, different from the antibiotic chlortetracycline heretofore produced from organisms of that genus.

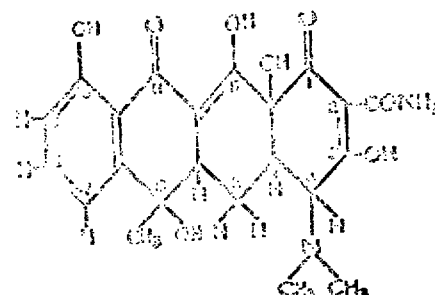
This substance was first produced in good yield and relatively high concentration in a fermentation medium otherwise conventional but substantially free from chloride ions, using as the organism a strain of *Streptomyces aureofaciens* found in soil. Mutants of this organism permit production of the antibiotic substance in higher concentration in a

medium containing a substantial content of chloride ions.

So far as we are aware all micro-organisms that produce both tetracycline and chlortetracycline by fermentation in an aqueous nutrient medium are of the genus *Streptomyces*. The species *S. aureofaciens* which produces chlortetracycline in fermentation media in which chloride ions are present, as well as the numerous naturally occurring and artificially induced mutants thereof, are preferably used since they produce tetracycline when allowed to ferment in media which are substantially free from chloride ions. Thus the invention is not particularly concerned with the use of any particular species of *Streptomyces* except that it should be one which is capable of producing both chlortetracycline and tetracycline by fermentation.

The newly discovered antibiotic substance has been named tetracycline as we have discovered that it possesses the structure which was first postulated as being a molecular structure common to chlortetracycline and oxytetracycline (Brunings et al; J.A.C.S., Volume 74; 4976-4977, October 5, 1952). Its freedom from chlorine differentiates it at once from chlortetracycline, and its behaviour in other respects demonstrates differences from oxytetracycline.

Tetracycline has the empirical formula $C_{22}H_{34}N_2O_8$ and the following structural formula:



[Price 3s. 6d.]

Tetracycline as the free base is amphoteric in character and forms salts with both acids and bases. For example, it forms addition salts with organic and inorganic acids and can be obtained in the form of the hydrochloride, hydrobromide, sulphate, borate, nitrate, phosphate, ascorbate, citrate, succinate, acetate, sulphamate and other acid-addition salts of a similar nature. Tetracycline also forms monobasic and dibasic salts with bases, as illustrated by bases of the alkali or alkaline-earth metals or as further illustrated by ammonium and amine bases. Specific examples are the salts with calcium, barium, strontium, magnesium, ethylene diamine, triethylamine, piperidine, potassium, and lithium. Salts, or more properly complex salts, are also formed by tetracycline with basic salts of boric acid as illustrated by sodium borate.

Salts of the above described types are of value in isolation and purification procedures, since they can be by neutralization or metathesis be converted to the free amphoteric material or converted from one form to the others. Such salts also possess antiseptic and therapeutic properties. The solubility of tetracycline in water varies with the pH becoming greater as the pH is raised or lowered from approximately 6. The form which is least soluble in water at an intermediate pH is referred to as isoelectric, or amphoteric material or as free base. It has been found by *in vitro* tests that these acid and basic salts as well as the isoelectric material or free base are effective against a large number of bacteria including both Gram positive and Gram negative bacteria.

The acid salts of tetracycline may be readily prepared by dissolving the free base in water or other suitable solvent acidified with an organic or inorganic acid. Similarly the basic salts may be readily formed by adding an organic or inorganic base to a solution of the free base.

Tetracycline can be distinguished from previously known antibiotics in many ways. A chemical analysis of the free base shows that the antibiotic contains the following elements: carbon, hydrogen, nitrogen and oxygen. Since it does not contain sulphur or chlorine, it is distinguished from many others and particularly from chlortetracycline which contains chlorine.

As is characteristic of many organic compounds, the properties of crystalline tetracycline are somewhat dependent upon the degree of purity and the manner in which the crystals are formed. When precipitated from solutions containing water, the free base precipitates as the trihydrate, which may be dried to the anhydrous form. Normally the free base is used in the trihydrate form, as the anhydrous form is quite hygroscopic and hydrates readily in the presence of moisture.

When recrystallized from a methanol-water solution, at room temperature, tetracycline crystallizes as a trihydrate. One crystalline phase of the trihydrate was yellow in colour and equant to tabular in habit. The refractive indices are found to be: $\alpha = 1.530 \pm 0.003$; $\beta = 1.646 \pm 0.003$; $\gamma = 1.798 \pm 0.003$. The optical angle $2V$ was found to be $80-85^\circ$ and the optical sign was found to be positive.

The optical rotation of highly pure crystalline tetracycline free base in methanol was determined

(α) $29^\circ \text{C} = -234^\circ$ (0.82% in methanol)

D

Tetracycline is soluble in methanol and beta-methoxyethanol (about 200 milligrams per milliliter) and somewhat soluble in ethanol and n-butanol, is slightly soluble in water and relatively insoluble in such solvents as ether, benzene and petroleum ether. Tetracycline hydrochloride is soluble in water to the extent of 100 to 200 milligrams per milliliter, quite soluble in methanol and ethanol and slightly soluble in 2-methoxyethanol and n-butanol but is relatively insoluble in other common organic solvents. The calcium salt of tetracycline is soluble in water to the extent of about 890 gammas per cc. at pH 8.55 and the magnesium salt is soluble in water to the extent of about 1,000 gammas per cc. at pH 7.0. In the following Table I are listed approximate solubilities of tetracycline trihydrate of high purity in various solvents.

TABLE I

Solvent	Solubility in 100 ml
Methanol	at least 3 g
Ethyl acetate	25 mg
Chloroform	20 mg
Water	less than 10 mg
Benzene	less than 10 mg
Diethyl ether	less than 10 mg

The melting point of anhydrous crystalline tetracycline, as for many other organic materials, depends on a number of factors including the degree of purity and the manner in which the crystals are heated. Crystals of highly pure anhydrous free base on a microscope slide were placed on a hot stage at 100°C . The temperature was increased at the rate of 2° per minute until a temperature of 145° was reached and then at 1° per minute until the crystals melted. When heated under these conditions, the melting point was in the range of $140^\circ-163^\circ \text{C}$. with decomposition.

A sample of the trihydrate in a capillary melting point tube was placed with a thermometer in a test tube containing a liquid at $150^\circ-155^\circ \text{C}$. This test tube was immersed

in a bath of the same material at 160°—165° C., which was then heated with stirring so that the temperature rose 1—2° C. per minute. The sample expanded slowly when the inner tube temperature rose to 160°—165° C. There was no darkening but a yellow colour developed. When the temperature reached 170—175° C., darkening started to take place. This became more pronounced with a further increase in temperature. When a sample of the anhydrous free base was subjected to the same treatment, similar results were obtained.

The infra-red spectrum of free anhydrous tetracycline mulled in hydrocarbon oil shows a strong OH or NH band at 3300—3400 cm^{-1} ; a possible amide carbonyl near 1655 cm^{-1} ; a possible C=C stretching frequency near 1613 cm^{-1} ; a possible NH bending vibration near 1580 cm^{-1} and other characteristic maximums near 703 cm^{-1} , 722 cm^{-1} , 785 cm^{-1} , 862 cm^{-1} , 950 cm^{-1} , 1178 cm^{-1} and 1228 cm^{-1} . The infra-red spectrum of tetracycline shows it to be related to chlortetracycline but the two compounds can be readily distinguished by means of their absorption spectrum. For example, chlortetracycline shows a strong doublet in the vicinity of 950 cm^{-1} , while tetracycline shows only a weak point of inflection and while chlortetracycline shows a strong triplet in the vicinity of 800 cm^{-1} , tetracycline shows only a weak point of inflection.

The ultra-violet absorption of both the free base and the hydrochloric acid salt of tetracycline was determined by measuring the optical densities of methanol solutions versus a methanol blank at various wave lengths (Beckman Quartz Spectrophotometer—Model DU). A solution of 1.0 mg of the free base in 50 ml of methanol gave the following maximum and minimum values:

1%
E at 235 μ = 188
1 cm

1%
E at 269 μ = 271
1 cm

1%
E at 298.5 μ = 123
1 cm

1%
E at 365 μ = 272
1 cm

The ratio of E at 269 to that at 365 is 1.00

and is characteristic of the purified antibiotic.

A solution of 1.10 mg of tetracycline hydro-

chloride in 50.0 ml of methanol gave the following maxima and minima:

1%
E at 234 μ = 179
1 cm

1%
E at 270 μ = 301
1 cm

1%
E at 298.5 μ = 131
1 cm

1%
E at 363 μ = 274
1 cm

The ratio of E at 270 to that at 363 is 1.10

and is characteristic of the antibiotic.

Tetracycline can be distinguished from other antibiotics including antibiotics produced by micro-organisms which are species of *Streptomyces* by means of Craig counter-current distribution studies. This technique is described in Craig, L.C., Journal of Biological Chemistry, Volume 150, page 33 (1943). For example, when tetracycline is distributed in a 49 plate Craig apparatus using the system n-butanol - 2.5% aqueous acetic acid, the highest concentration of tetracycline is in tube 18. Utilizing the same system, the highest concentration of chlortetracycline is in tube 28, the highest concentration of chloramphenicol in tube 47, and the highest concentration of oxytetracycline is in tube 16. These studies were made at room temperature. The relation to oxytetracycline is close, but the consistency of results shows that the difference of two plates is significant.

Tetracycline can also be distinguished from oxytetracycline or chlortetracycline by means of the Ehrlich reagent. In carrying out this test, a solution of each of the three antibiotics was made up at the concentration of 5 mg in 5 ml of 1.2 N hydrochloric acid. Then one milliliter of a 2% (w/v) solution of p-dimethyl-amino-benzaldehyde in 1.2 N hydrochloric acid was added to each solution and allowed to stand overnight. The solution of chlortetracycline turned canary yellow, while in the oxytetracycline solution a bluish-green precipitate formed with a similarly coloured supernatant liquor. The tetracycline solution turned a deep yellow with a trace of orange. When both the reagent and 5 mg of chlortetracycline were added to the above solution of oxytetracycline, a bluish-green precipitate and supernatant liquor were obtained just as upon the addition of the reagent alone. However, when both the reagent and 5 mg of tetracycline was added to the oxytetracycline solu-

tion, there was no precipitate and no colour change.

- 5 Tetracycline can be further differentiated from both chlortetracycline and oxytetracycline by the difference in colour in the presence of sulphuric acid. When chlortetracycline is dissolved in concentrated sulphuric acid (approximately 25 mg/ml) a purple solution is formed which changes after a few seconds to greenish-black. When oxytetracycline is dissolved in concentrated sulphuric acid, a stable characteristic cherry-red solution is obtained (Monastero, R. et al, Journal of American Pharmaceutical Association, Scientific Ed., 40: 241—245, May 1951). However, when tetracycline is dissolved in the same acid, the solution has

a stable violet colour.

The antibacterial spectrum is characteristic of the antibiotic and is another aid in distinguishing the antibiotic as well as being indicative of the organisms against which the antibiotic is effective. The following Table II shows the relative amounts in mg/ml of tetracycline hydrochloride, chlortetracycline hydrochloride, oxytetracycline hydrochloride and chloramphenicol required to inhibit a number of representative organisms. The method used was the agar-dilution streak method of Wakeman and Reilly (Ind. Eng. Chem. Anal. Ed., Volume 17: 556—558, 1945). These data show that tetracycline is a distinct antibiotic substance.

TABLE II

Test Organism	A	B	C	D
<i>Staphylococcus aureus</i> PCI 209—P	0.292	6.25	0.39	0.58
<i>Staphylococcus aureus</i> PCI 1248—A	0.39	6.25	0.78	0.78
<i>Streptococcus pyogenes</i> ATCC 8663	0.29	1.17	0.24	0.24
<i>Streptococcus mitis</i> ATCC 6249	0.147	2.34	0.147	0.292
<i>Streptococcus faecalis</i> ATCC 7080	0.39	6.25	0.78	1.95
<i>Micrococcus flavus</i> PCI 16	0.292	1.56	0.39	0.38
<i>Diplococcus pneumoniae</i> ATCC 6301	0.098	2.34	0.147	0.292
<i>Serratia lutea</i> ATCC 9341	0.147	1.56	0.39	0.39
<i>Bacillus subtilis</i> ATCC 6633	0.195	6.25	0.39	0.39
<i>Escherichia coli</i> ATCC 9637	1.45	12.5	1.17	1.17
<i>Klebsiella pneumoniae</i> PCI 602	0.29	1.17	0.58	0.58
<i>Aerobacter aerogenes</i> ATCC 8508	1.17	3.12	2.34	1.56
<i>Proteus vulgaris</i> ATCC 6380	4.6	3.12	3.9	3.12
<i>Pseudomonas aeruginosa</i> ATCC 9027	25.0	> 100.	25.0	15.0
<i>Salmonella schottmulleri</i>	3.12	3.12	2.34	3.12
<i>Salmonella typhosa</i>	1.17	4.6	2.34	1.56
<i>Burkella bronchiseptica</i> ATCC 4517	0.292	6.25	0.78	1.17
<i>Mycobacterium tuberculosis</i> ATCC 607	0.147	6.25	0.195	0.29

TABLE II—continued

Test Organism	A	B	C	D
<i>Mycobacterium friedmanii</i> ATCC 14	0.122	6.25	0.24	0.292
<i>Mycobacterium smegmatis</i> ATCC 351	0.073	12.5	0.147	0.292
<i>Mycobacterium smegmatis</i> ATCC 10143	0.073	6.25	0.147	0.122
<i>Mycobacterium</i> sp. ATCC 9820	0.58	6.25	0.58	2.34
<i>Candida albicans</i> ATCC 10231	> 100.	> 100.	> 100.	> 100.

A = Chlortetracycline hydrochloride

B = Chloramphenicol

C = Tetracycline trihydrate

D = Oxytetracycline hydrochloride

P.C.I. = Penicillin control Immunology

A.T.C.C. = American type culture control

Table III sets forth the cultural characteristics of two isolates derived from the culture of *Streptomyces aureofaciens* hereinbefore referred to which have been used in the pro-

duction of tetracycline. These characteristics are identical with those exhibited by a known culture of *Streptomyces aureofaciens*.

TABLE III

10	Glucose broth	---	yellowish growth settled on the bottom, acid reaction
	Lactose broth	---	yellowish growth settled on the bottom, no acid reaction
	Glycerin broth	---	yellowish growth settled on the bottom, no acid reaction
15	Sucrose broth	---	yellowish growth settled on the bottom, acid reaction
	Limus milk	---	surface growth, slight clearing, no pH change
	Gelatin stab	---	growth good at the surface, no liquefaction, yellow pigment
20	Asparagine-meat extract agar	---	slight yellow pigment, good growth, aerial hyphae, white becoming grey
	Campbell-Dox agar	---	poor growth, flat colorless mycelium, no aerial hyphae, no soluble pigment
25	Emerson agar	---	heavy growth, brownish mycelium, no aerial hyphae, back brownish, slight soluble brown pigment
	Nutrient glucose agar	---	same as Emerson
30	Glycerol agar	---	growth heavy, white aerial hyphae, turning grey, back greenish yellow, slight soluble yellow pigment
	Starch agar	---	flat colorless mycelium, no aerial hyphae, no soluble pigment, hydrolysis
35	Calcium melate agar	---	growth heavy, aerial hyphae white turning grey, pigment brownish

The strains or mutants of *Streptomyces aureofaciens* which are preferred in carrying out the present invention are those which produce relatively high yields of tetracycline in the fermentation medium, particularly those which will produce more than 500 mcg/ml. One strain when grown on Wellesman agar (Journal Bacteriology: volume 7; 339-341, 1952) exhibits heavy mycelial growth which is off-white at first, becoming yellow and which is gradually covered with a powdery white aerial mycelium, which later sporulates. Old slants exhibit a jet-black coloration with small patches of white mycelium. This black growth, which has not heretofore been reported in cultures of *Streptomyces aureofaciens* and is not a characteristic of the species, consists of a mass of bodies in short chains which are easily disrupted. These bodies are variable in size and shape, ranging approximately from 0.5 up to 4.5 μ and varying in shape from oval to slightly angular globose-spherical. The average size is somewhat greater than those in *Streptomyces aureofaciens* (NRRL-2209) and there is greater variation in size and shape.

Both natural and induced mutants of *Streptomyces aureofaciens* may produce tetracycline in varying amounts, in addition to chlortetracycline. By selection of a natural or induced mutant which produces a comparatively high ratio of tetracycline to chlortetracycline, and propagating the organism under controlled conditions, a comparatively large yield of tetracycline may be obtained.

Among mycologists the classification of micro-organisms is frequently a difficult problem, and different mycologists may arrive at different classifications for the identical organism. The appearance of *Streptomyces aureofaciens* under different conditions may differ widely. Morphologically indistinguishable strains of *Streptomyces aureofaciens* vary markedly in their production of tetracycline and chlortetracycline, and in the ratio between the two.

Tetracycline has been produced by the growth of many natural isolates of *Streptomyces aureofaciens*. The gross appearance, and detailed examination of these isolates vary considerably.

Natural and induced mutants or variants have been obtained with growth colours in various shades of red, orange, yellow, green, brown and grey; they have varied in growth habit, especially in liquid media in shake flasks from filamentous to short length hyphae elements giving a pasty yeast-like appearance. The mutants may differ in vigour and rate of growth so that maximum growth in submerged cultures may occur over a period ranging from about a day to over a week. The rate of growth is not necessarily co-extensive with antibiotic production.

We have found that during the fermentation, it is desirable to grow the organisms herein-

before referred to under submerged aerobic conditions with suitable aeration and agitation, as for example, in a flask on a shaker machine or in a stirred fermentor equipped with a sparger for inducing a stream of air continuously. The temperature does not appear to be critical within the range of 25°-35° C. although the range of 30°-33° C. is preferable. The initial pH of the medium should be close to neutrality, although some of the antibiotic is produced in media with initial pH values as low as 3.5 or as high as 8.5.

A fermentation medium suitable for the production of tetracycline contains a source of assimilable carbon, such as an assimilable carbohydrate, a source of assimilable nitrogen, inorganic salts such as phosphates and magnesium salts, and sources of the usual minor elements. Buffers are usually included.

The carbon source may be either a soluble carbohydrate such as sucrose or an insoluble one such as starch, for example, corn starch. Dextrin may also be used. Although sucrose, a soluble sugar, is quite satisfactory, lactose and glucose, two other soluble sugars, are relatively poor. The amount of carbon source may vary widely from about 0.5 to 10.0% by weight of the total weight of the fermentation medium.

As a source of assimilable nitrogen the inorganic ammonium salts are suitable. Among these are ammonium sulphate and ammonium phosphate. It is also possible to use organic sources of nitrogen such as the amino acids and various proteinaceous natural materials.

Included among the inorganic salts found advantageous are phosphates which may be present either as ammonium phosphate or as metallic phosphate such as potassium phosphate, magnesium sulphate and a source of potassium if this is not already present, for example, as potassium phosphate.

Addition of small amounts of the heavy metals is desirable unless they are present in the other ingredients. Among these trace elements which may be present as impurities or added are copper, zinc, manganese, iron and chromium.

Among the buffering agents are calcium carbonate and salts of organic acids such as the citrates, acetates and lactates which are useful in maintaining the pH within the proper range. In addition, the organic acids may serve as sources of carbon in the metabolism of the micro-organism. The use of a defoaming agent is desirable in large scale fermentors, even though in this fermentation foaming is not a particularly difficult problem and is readily controlled by the use of conventional defoaming agents such as octadecanol in kerd oil or other suitable commercial defoamer.

Micro-organisms of *Streptomyces aureofaciens* which produce chlortetracycline may be used to produce tetracycline by reducing the available chloride ion in the fermentation

medium to a minimum. There is some concomitant formation of chlorotetracycline if a substantial amount of available chloride ion is present, and to recover a product free from chlorotetracycline, it may then be necessary to employ selective recovery steps which exclude that substance from the recovered product.

There are various ways of controlling the chloride ion content. One way is to regulate the chloride in an inactive complex. Successful results have been obtained by the use of distilled water and the use in the medium of constituents which do not contain chloride. So-called synthetic media, of which examples are given below, serve that purpose. Many natural materials often used in fermentation media contain chloride. Their use is possible nevertheless if they are first dechlorinated by treatment with ion exchangers or other suitable means of removing chloride ion.

We have devised a synthetic medium which is substantially free from chloride, which produces superior yields, is easy to prepare, is lower in cost and is consistent in composition. This medium contains sucrose as the carbohydrate, ammonium sulphate as a nitrogen source, potassium phosphate as an inorganic salt, magnesium sulphate, calcium carbonate, sodium citrate and acetic acid as buffering agents and copper sulphate, zinc sulphate and manganese sulphate in small quantities as sources of trace elements. Substantially chloride-free grades of these materials are selected, and distilled water is used, so that this medium contains not more than 1 ppm of chloride.

The composition of this synthetic medium is:

	Sucrose	30 g/l
	(NH ₄) ₂ SO ₄	3.1 "
40	Na ₂ C ₆ H ₅ O ₇ ·5H ₂ O	1.0 "
	MgSO ₄ ·7H ₂ O	0.25 "
	K ₂ HPO ₄	0.57 "
	CaCO ₃	1.0 "
	CH ₃ COOH	0.1 ml
45	CuSO ₄ ·5H ₂ O	3 ppm
	ZnSO ₄ ·H ₂ O	50 ppm
	MnSO ₄ ·4H ₂ O	2.5 ppm

Inoculum for fermentation may be prepared from growth obtained on slants inoculated with a strain of *Streptomyces aureofaciens*. A suitable medium for the slants is Williams agar having the following composition:

	Glucose	10 g/l
	Peptone	5 "
55	K ₂ HPO ₄	1 "
	MgSO ₄ ·7H ₂ O	0.5 "
	Agar	20. "

The slant growth may be transferred to shaker flasks which may be used as small scale laboratory fermentations or for the production of

inoculum for inoculating larger fermentations.

For large scale tank fermentation, the slant growth is used to seed a suitable liquid medium in a shaker flask which is shaken on a reciprocating shaker machine at temperatures ranging from 25°-35° C., preferably at 30°-33° C. A second shaker flask stage is usually employed in conjunction with submerged tank fermentations in order to increase the volume of the slant inoculum equal to 1.5% to 2.5% of the liquid volume in the tank. The reaction of the shaker flask medium is within the range of pH 6.5 to 7.5 initially and, as growth occurs, a continuous drop in the pH value is observed with values as low as 3.8 being recorded. However, a physiological age as indicated by a pH value of 5.0 is apparently the most favourable period for transferring the inoculum.

The shaker inoculum, equal to 1.5% to 2.5% of the liquid volume in the tank, is aseptically transferred to the fermenter and grown for about 2 to 3 days with continuous agitation and aeration. Air rates equal to 0.5 to 1.5 volumes of free air per volume of liquid medium may be used depending upon the size of the fermentor. Foaming of the broth can be controlled by the sterile addition of an anti-foam agent such as lard oil containing 2% octadecanol.

RECOVERY AND PURIFICATION

The tetracycline may be recovered in various ways from the fermentation medium. For example, the fermented material may be added directly to animal feeds, thus providing a product particularly suited for stimulation of growth and/or the control of disease. When the mycelia and insolubles are not desired, the fermentation medium may be filtered with or without acidification, and the clear liquor added to drinking water or animal feeds for the same purposes. If desired, the liquor containing the tetracycline may be concentrated under reduced pressure or by spray drying and the antibiotic used in the crude form for growth stimulation or the control of treatment of disease in man, animal and fowl.

When it is desirable that the tetracycline be in a more highly purified state it may be recovered from the fermented medium by a variety of procedures which include one or more of the steps, in various orders, of adsorption, elution, solvent extraction, with or without emulsions, e.g., "Wet" extraction in the form of insoluble salts of calcium, barium, strontium or magnesium salt at a pH above 6.8 or as insoluble salts with alkalis or sulphonic acids of the wetting agent or dye type; or it may be extracted into solvents with emulsions such as sulphur or sulphurates of the wetting agent or dye type. The tetracycline may be separated from the crude mash by adjusting to a pH not exceeding 3, and the tetracycline will pass into the aqueous layer and the insoluble solids may be separated and

removed. Alternatively the nutrient medium may contain a source of calcium, strontium, barium or magnesium cations thus enabling the antibiotic activity to be precipitated as it is formed. Tetracycline may be precipitated in aqueous solutions as precipitate with calcium, strontium, barium or magnesium cations at a pH above 6.5. This precipitate may be extracted with acidified water or with organic solvents such as the alcohols, alkoxyalkanols or ketones. The extraction may be under acid, neutral or basic conditions. Similarly, the aqueous solution containing tetracycline may be extracted by aqueous layer immiscible organic solvents such as aqueous layer immiscible lower alcohols, alkoxyalkanols, esters and ketones. Of known solvents such as butanol, chloroform, and ethyl acetate, butanol is preferred for the extraction of tetracycline. Certain alcohols such as isopropanol which are water miscible, but are immiscible with a saline solution are also useful. The addition of a water-soluble organic-layer-insoluble salt, such as an ammonia or amine salt, or an alkali metal halide, or sulphates, assists in the extraction procedure.

The tetracycline may be caused to precipitate from aqueous solutions by the addition of water soluble salts such as ammonia or amines or alkali metal halides and sulphates. The salting out may be assisted by a small portion of an organic solvent.

Chlortetracycline if present in the fermentation mash normally comes through these procedures with the tetracycline. The chlortetracycline may be permitted to degrade by maintaining the mixture under such conditions that the tetracycline is more stable, or the chlortetracycline may be reduced to tetracycline.

We have also devised a novel recovery method which is highly effective both from the standpoint of recovering a product free from inactive impurities and from the standpoint of recovering a product free from other substances concomitantly formed in the medium. This recovery method yields a crystalline product of high purity from both standpoints. In its main features, it involves using quaternary ammonium salts which selectively precipitate tetracycline from the broth, at an alkaline pH (pH 8 to 11) as quaternary ammonium salts of tetracycline. Such a salt, after filtration, is then slurried with a small amount of water and a relatively large amount of chloroform, whereby the quaternary ammonium salt of tetracycline is dissolved in the chloroform phase of the slurry. The water and chloroform phases are then preferably separated to remove any impurities dissolved in the water. The tetracycline is then extracted with an aqueous acid solution at a pH of about 1 to 2.5, with formation of the acid salt of tetracycline which goes into solution in the water phase, from which it precipitates as a crystalline product upon increase of the pH to the

range from 3 to 7, precipitation starting at the lower value.

The quaternary ammonium salts that are particularly useful in this recovery method are alkyl trimethylammonium chlorides and dialkyl dimethylammonium chlorides wherein the alkyl group contains from 8 to 18 (inclusive) carbon atoms.

In order that the invention may be fully understood it will now be described with reference to the following specific examples. Examples I to III illustrate the production of the antibiotic, tetracycline, by fermentation under submerged aerobic conditions: the same media may be used for fermentation in shaker flasks on a smaller scale or in tanks on a larger scale. Examples IV to IX illustrate recovery and purification.

EXAMPLE I

A corn steep liquor medium was prepared in the following manner. Corn steep liquor of approximately 50% solids content (w/v) was diluted with distilled water to give a final concentration of 2%. The resulting diluted material was passed through a column packed with 30 litres of a mixture consisting of 2 volumes of Amberlite IR45, an anionic exchange resin, and 1 volume of Amberlite IRC50, a cationic exchange resin (the word "Amberlite" is a Registered Trade Mark). Collection of the effluent did not start until the resistivity had risen to 5,000 ohm-centimeters and continued until the resistivity had dropped below 3,000 ohm-centimeters. To 1,000 ml of the deionized corn steep liquor solution there was added the following components for the preparation of a medium suitable for the propagation of inoculum and for the production of tetracycline to produce a medium containing about 17 ppm of chloride, although a lower chloride content is preferred:

Sucrose	30.0 grams
K ₂ HPO ₄	15.0 grams
(NH ₄) ₂ HPO ₄	5.0 grams
MgSO ₄ ·7H ₂ O	2.0 grams
CaCO ₃	1.0 grams
KBr	0.5 grams
ZnSO ₄ ·7H ₂ O	0.05 grams
CuSO ₄ ·5H ₂ O	3.0 milligrams
MnSO ₄ ·4H ₂ O	2.5 milligrams

For the preparation of an inoculum, 50-milliliter volumes of the above medium were distributed into 250-milliliter Erlenmeyer flasks and 400-milliliter volumes into 2-litre flasks. A dry loopful of spores from the surface growth of the tetracycline organism grown on slanted semi-solid medium was used to seed the 50-milliliter volumes which were shaken at 30° C. for 72 hours on a reciprocating shaking machine (82 strokes per minute with a 2.875 inch displacement). At the end of this time 1.0 ml of the resulting mycelial suspension was used to inoculate 400 ml. of medium which

was shaken for an additional 28 hours. Four 400-ml. volumes were pooled aseptically and used to seed the fermentor.

- The fermentation medium was prepared in 25-gallon fermentors as follows: The components, at the concentrations described above were dissolved in 11.5 gallons of the deionized corn steep liquor solution for a final total volume of 15.8 gallons. The medium was sterilized at 121° C. by injecting steam at 15 PSI gauge directly into the batch for 30 minutes. The volume gained from the steam condensate provided the required difference between the initial 11.5 gallons of deionized corn steep liquor-sugar-salts solution and of the final 15.8 gallons required. Upon cooling to 30° C., the mixture was seeded with the four pooled 400-ml. volumes of inoculum previously described, agitated and aerated at a rate of 1.6 volumes of air per volume of medium for 38 hours. At this time the reaction of the broth which was initially at pH 5.9 had dropped to 4.1. The broth assayed 20 mcg/ml. of antibiotic, using oxytetracycline as a standard and contained a substantial amount of tetracycline.

EXAMPLE II

- Fifty gallons of the inoculum medium described in Example I were made up in a 125-gallon stainless steel inoculum tank and sterilized for 30 minutes at 121° C. The temperature was brought down to 33° C. and the batch seeded with 400 ml. of a 72-hour old shaker flask inoculum grown in the same medium. The tank was aerated with 1.0 volume of air per volume of medium and agitated for a period of 20 hours, at which time a pH of 5.05 was attained and the broth used to seed a fermentor.

- The fermentation medium, of very low chloride content, had the following composition:

	Sucrose	40.0	grams
	CaCO ₃	1.0	grams
	MgSO ₄ ·7H ₂ O	0.25	grams
45	KH ₂ PO ₄	0.10	grams
	K ₂ HPO ₄	0.10	grams
	(NH ₄) ₂ SO ₄	3.3	grams
	Sodium citrate	2.0	grams
	ZnSO ₄ ·7H ₂ O	0.04	grams
50	MnSO ₄ ·4H ₂ O	0.01	grams
	K ₂ CrO ₇	0.016	mg.
	Acetic acid	0.5	ml.

Distilled water was used to bring the final volume to 1,000 ml.

- Six hundred gallons of the above medium was made up in a 1,000-gallon stainless steel tank and sterilized for 25 minutes at 121° C. Upon cooling to 30° C., the 50 gallons of the previously described contents of the inoculum tank was introduced aseptically and the entire contents agitated and aerated at the rate of 0.68 volumes of air per volume of medium per minute for a period of 71 hours at which time

the broth showed a potency of 92 mcg./ml.

EXAMPLE III

The medium for inoculum was prepared (50 ml. in 250-ml. Erlenmeyer flasks) with the following ingredients:

Sucrose	30.	g/l	
Sodium citrate	1.0	g/l	70
(NH ₄) ₂ SO ₄	3.3	g/l	
MgSO ₄ ·7H ₂ O	0.25	g/l	
KH ₂ PO ₄	0.10	g/l	
K ₂ HPO ₄	0.10	g/l	
CaCO ₃	1.0	g/l	75
Acetic acid	0.4	ml.	
Soy grits	0.5	grams	
Trace element solution	1.0	ml.	

Distilled water used to bring the final volume to one liter. The solution of trace elements has the following composition:

CuSO ₄ ·5H ₂ O	0.3	g/10-ml
ZnSO ₄ ·7H ₂ O	5.0	
MnSO ₄ ·4H ₂ O	0.25	

(The pH was adjusted to 6.2 with NaOH)

The materials were added in the order given and autoclaved. The flasks, after cooling, were inoculated from a slant of a *Streptomyces aureofaciens* culture and shaken at 30°—33° C. for 48 hours on a reciprocating shaker (3.5-inch stroke and 97 cycles/minute).

For production of tetracycline the following medium was prepared:

Sucrose	30	g/l distilled water	
MgSO ₄ ·7H ₂ O	0.25		95
KH ₂ PO ₄	0.15		
CaCO ₃	1.5		
Citric acid	11.5		
NH ₄ OH	10	ml	
Trace element solution	1.0	ml (as previously described)	100

In each 250-ml flask there was placed 50 ml of the medium and after sterilization, 2.5 ml of inoculum was transferred aseptically to each flask. The initial pH was between 6.0 and 7.0. After three days incubation on a rotary shaker at 250 rpm. and 30° C. the assays in ten individual shaker flasks averaged 610 mcg/ml against a tetracycline standard of 1,000 mcg/ml for the pure material with *E. coli*. The assay against tetracycline standard was 610 mcg/ml. With a chloride content of 0.6 ppm, the total potential amount of chlortetracycline produced was 8 mcg/ml or about 1.4% of the total antibiotic.

EXAMPLE IV

This is an example of recovery of tetracycline from a fermentation medium. To 155 gallons of broth prepared substantially as in Example II and assaying 68 mcg/ml there was added 5600 grams of oxalic acid to solubilize

the antibiotic. The pH was adjusted to 3.5 with 4600 ml of concentrated ammonium hydroxide and the mixture filtered with 15 pounds of filter-aid to remove the mycelium and the precipitated calcium oxalate. Thirty gallons of a 5% solution of N-(lauroyl col-

amino formyl-methyl) pyridinium chloride, in ethyl acetate was added to the filtered broth, and the mixture adjusted to pH 8.5 with 2200 ml of 50% sodium hydroxide. The mixture was agitated for 20 minutes and allowed to settle.

The ethyl acetate layer of 82 litres containing the antibiotic was separated and extracted three times using 1500 ml of water and adjusting to pH 1.7 with hydrochloric acid each time. Sulphuric acid may be used. The extracts were pooled and adjusted to pH 7.8 with 6.5 ml of 4N ammonium hydroxide to form the relatively insoluble isoelectric material. The mixture was filtered and the cake dried in a vacuum desiccator. The weight of crude material was 21 grams; the assay was 417 mcg/mg. A countercurrent distribution of the material showed some 75% of the antibiotic present to be tetracycline.

EXAMPLE V

A fermentation medium containing more than 130 mcg/ml of tetracycline was acidified to pH 3.5 with concentrated sulphuric acid to solubilize the antibiotic and filtered after the addition of a filter aid. The volume of filtered broth was 1070 gallons. To this broth there was added 2140 ml of "Arquad 16," and the pH of the broth was brought up to 10.0 by the addition of 6160 ml of 50% aqueous sodium hydroxide. ("Arquad 16" is a mixture of alkyl trimethylammonium chloride and dialkyldimethylammonium chloride in which the alkyl groups consist of 90% hexadecyl, 6% octadecyl, and 4% octadecenyl, in isopropanol. The alcohol constitutes about 50% of the preparation). After the "Arquad" was added, the pH was adjusted to 8—11, to form the quaternary ammonium salts of tetracycline which precipitate out in this pH range. After the addition of 20 pounds of filter-aid, the broth was filtered and the solids thus obtained washed with water. Approximately four-fifths of this washed filter cake was slurried with 12 gallons of methanol solvent and sufficient concentrated hydrochloric acid added to bring the pH to 1.8 thereby increasing the solubility of the tetracycline. After stirring for 15 minutes the mixture was filtered and the solids washed with methanol until substantially free of colour. The total volume of extract and wash was 92 litres. To the combined extract and wash there was added sufficient 10 N aqueous sodium hydroxide to bring the pH to 6.8, thereby forming the relatively insoluble free base which precipitated. The solids were filtered and air-dried. There was obtained 1545 grams of crude amorphous isoelectric tetracycline adhydrate assaying 296 mcg/mg.

EXAMPLE VI

2140 ml of "Arquad 16" was added to 1070 gallons of filtered fermentation broth at pH 3.5 and the pH brought up to pH 10. Twenty pounds of filter-aid was added and the mixture was filtered and the solids washed with water. A one kilogram aliquot portion of the washed filter cake was slurried with 3300 ml of chloroform and the pH adjusted to 10.5. This pH value may be in the range of 8—11. After stirring for 15 minutes, the solids were filtered and reslurried twice in succession with a mixture of 150 ml water and 3300 ml chloroform. The pooled chloroform extracts containing the antibiotic and from which the aqueous phase had been separated were then stirred with 600 ml of 0.4N sulphuric acid and the pH adjusted to 2 with 4N sulphuric acid. After 5 minutes stirring, the aqueous phase was separated and filtered. A 340-ml aliquot of this acid concentrate was adjusted to pH 5.3 and allowed to stand overnight in the refrigerator. The crystals were filtered, washed with ice water, and dried in vacuo over anhydrous calcium chloride. Nineteen and one-half grams assaying 629 mcg/mg were obtained. There was no detectable amount of chlortetracycline present.

This recovery process is preferred as it is efficient in recovery and produces crystalline material. The filter cake comprising the precipitated quaternary ammonium salts of tetracycline is extracted with chloroform which is wet as a result of water in the chloroform or in the cake or as a result of being added. If chloroform is used under anhydrous conditions, lower yields are obtained. Preferably the filter cake is repeatedly extracted and the aqueous phase is preferably discarded. The chloroform extract is acidified with an inorganic acid to a pH preferably in the range of about 1—2.5 to convert the tetracycline salts to corresponding acid salts of tetracycline which are water soluble. Before or during addition of the acid water is added to form the two phases with the tetracycline acid salts in the aqueous phase. The aqueous phase is separated from the chloroform phase and the pH is raised to about 3 to 7 to form the relatively insoluble isoelectric material. Relatively low pH values are preferred, as purer crystals are obtained. The isoelectric material separates on standing as trihydrate crystals which can be separated readily by filtration and washed if desirable.

EXAMPLE VII

Two columns each containing 50 grams of "Amberlite IRC50" resin in the hydrogen cycle were buffered to pH 7 by passing through each at a rate of 10 ml/minute one litre of N sodium acetate-acetic acid buffer. "Amberlite IRC50" resin is a carboxylic cation exchange resin. Ten litres of filtered broth assaying 41 mcg/ml was adjusted to pH 7 with 4N aqueous sodium hydroxide and passed through one of the buffered "Amberlite

IRC50" column at a rate of 5 ml/minute. The following fractions of effluent were collected:

- 5 0—2 litres assaying 13.3 mcg/ml
 2—5 litres assaying 8.7 mcg/ml
 5—7 litres assaying 15.1 mcg/ml
 7—10 litres assaying 25.0 mcg/ml

10 The effluent from this column was passed through the second buffered column also at a rate of 5 ml/minute and fractions of effluent from the second column collected for assay.

- 15 0—2 litres assaying 16.9 mcg/ml
 2—4 litres assaying 12.4 mcg/ml
 4—6 litres assaying 7.5 mcg/ml
 6—10 litres assaying 7.2 mcg/ml

20 One litre of N hydrochloric acid in 60% methanol was passed through the first column at a rate of 10 ml/minute. The eluate which contained 74% of the activity in the filtered broth was concentrated *in vacuo* to approximately one-third its volume when the pH had dropped to 2.5. The pH was brought to 1.8 by the addition of a mineral acid and a filter aid was added and the material filtered in order to remove solids that separated during the concentration. The filtered solids were washed with water and the combined filtrate and washings brought to a pH of 7.8 by the addition of 4N sodium hydroxide. The solids that separated were washed and then dried *in vacuo*. There was obtained 1.375 grams of product assaying 78 mcg/mg.

EXAMPLE VIII

35 Tetracycline can be precipitated from aqueous solutions thereof at a pH above 7 by the addition of a mixture of barium chloride and magnesium chloride. For example, when 15 grams of barium chloride dihydrate and 2 grams of magnesium chloride hexahydrate was added to a solution of 3 grams of crude tetracycline in one litre of water at pH 2.5 and then aqueous sodium hydroxide added to bring the pH to 8.5, all of the activity was found in the precipitate, which is separated by filtration from the liquor containing impurities. When this precipitate was slurried with water and the pH brought to 1.5 with sulphuric acid and the mixture filtered to remove the insoluble barium sulphate, the resulting aqueous extract contained practically all of the microbiological activity originally present. This procedure may be used to concentrate tetracycline-containing

solutions and broths and to remove impurities.

EXAMPLE IX

Crystals of tetracycline free base were formed by dissolving crude amorphous tetracycline in water. This crude material assayed approximately 427 mcg/mg. The aqueous solution was adjusted to pH 3 by the addition of hydrochloric acid. The solution was then saturated with n-butanol and thereafter subjected to a counter-current distribution with nine separatory funnels utilizing equal volumes of n-butanol saturated with water which had been adjusted to pH 3 with dilute hydrochloric acid. After distribution, the middle tubes 4, 5, 6 and 7 were combined and the pH brought up to 7 by the addition of dilute sodium hydroxide solution. This mixture was concentrated under a vacuum of 40—50° C. The crystals that formed upon cooling were separated by filtration and dried over calcium chloride in a vacuum desiccator. The crystalline trihydrate product thus obtained assayed 780 mcg/mg.

ASSAY OF TETRACYCLINE

For the assay of tetracycline *E. coli* 9637 and Difco Nutrient agar ("Difco" is a registered trade mark) at pH 6.0 may be used to determine concentrations ranging from about 15 to 100 mcg/ml. *S. lutea* 9341 with Difco Penassay Seed agar adjusted to pH 6.0 is a more sensitive organism and may be used to determine concentrations ranging from about 5 to 50 mcg/ml. In all cases the dilutions were made in 0.1 M potassium phosphate buffer at pH 6.0 and applied in 0.1 ml volumes to S and S No. 420E 12.5 mm paper discs. All plates were incubated at 30° C. for 18 hours and zones of inhibition measured on a Quebec Colony Counter. Results may be read from a standard curve prepared on the same day, or a multiple dose method may be used. A sample of purified tetracycline was assigned an arbitrary value of 1,000 mcg/mg on an anhydrous basis. All values reported have been calculated against this standard unless otherwise noted.

TOXICITY OF TETRACYCLINE

Tetracycline shows little toxicity when administered to animals. The following Table IV shows the relative quantities of tetracycline, chlortetracycline and oxytetracycline required to kill mice following administration of a single dose by routes indicated. It is seen that the toxicity of tetracycline is comparable to those of chlortetracycline and oxytetracycline.

TABLE IV

Route of Administration	Tetracycline mg/Kg.	Chlortetracycline mg/Kg.	Oxytetracycline mg/Kg.
Intravenous	LD ₅₀ 145	LD ₅₀ 140	---
Intraperitoneal	LD ₅₀ 355	LD ₅₀ 365	LD ₅₀ 280
Subcutaneous	LD ₅₀ 400	LD ₅₀ 400	LD ₅₀ 340
Oral	LD ₅₀ 4250	---	LD ₅₀ 3750

ADMINISTRATION OF TETRACYCLINE

Animal protection tests using mice infected with *D. pneumoniae*, *K. pneumoniae* and *S. pyogenes* show that tetracycline when administered subcutaneously or orally, has activity comparable to that of oxytetracycline and chlortetracycline.

Tetracycline has been prepared in a purity in excess of 95% and with a negligible content of other antibiotic substances. An alternative form of the product comprises a significant content, not exceeding about one-half, of other compatible antibiotic substances either added to the preparation or formed concomitantly in the fermentation broth and co-crystallized in the recovery step, as when the above described butanol extraction or the extraction procedure of Example IV is employed. These products may be administered to humans and animals in the manner of known broad spectrum antibiotics, and in the conventional dosage forms appropriate to the desired route of administration.

What we claim is:—

1. A process of producing an antibiotic substance defined as tetracycline which comprises inoculating and aerobically fermenting an aqueous nutrient medium containing assimilable sources of carbon and nitrogen and mineral substances with a micro-organism of the genus *Streptomyces* which tends to produce chlortetracycline in the presence of chlorine ions, said method being characterized by controlling the chloride ion content of the nutrient medium so that the formation of the chlortetracycline is restricted and tetracycline is formed as the predominating antibiotic.

2. A method according to claim 1, in which the nutrient medium contains less than 1 ppm of available chloride ions.

3. A method according to either of claims 1 or 2, in which constituents of said medium normally containing available chloride ions have been treated with an ion exchange resin to remove said chloride ions.

4. A method according to any of claims 1 to 3, in which the nutrient medium is maintained at a pH from 3.5 to 8.5.

5. A method according to any of claims 1 to 4 in which the fermentation is carried out at a temperature of 25° C. to 35° C.

6. A method according to any of the preceding claims, in which the medium contains a source of calcium, strontium, barium, or magnesium ions whereby the antibiotic activity is precipitated as it is formed.

7. A method of recovering tetracycline from tetracycline-containing material which comprises forming a solution of said material, if not already in solution, and then adding a quaternary ammonium compound whereby the antibiotic is precipitated as a salt thereof.

8. A method according to claim 7, in which the quaternary ammonium compound is an

alkyl trimethyl ammonium chloride or a dialkyl dimethyl ammonium chloride in which each alkyl group contains from 8 to 18 carbon atoms.

9. A method according to either of claims 7 or 8, in which the precipitated quaternary ammonium salts are purified by solvent extraction.

10. A method according to claim 9, which comprises extracting the separated salts with chloroform in the presence of water, acidifying the chloroform extract to a pH of 1 to 2.5, adding water to form a chloroform phase and an aqueous phase containing the tetracycline, separating the aqueous phase, adjusting the pH of the aqueous phase to a pH of 3 to 7, and separating the resulting precipitated crystals of the antibiotic.

11. A method of recovering tetracycline from tetracycline-containing material which comprises forming a solution of said material, if not already in solution, effecting chromatographic adsorption of the antibiotic and eluting the latter.

12. A method of recovering tetracycline from tetracycline-containing material which comprises separating the antibiotic activity from the tetracycline-containing material as a precipitate with calcium, barium, strontium or magnesium cations, at a pH above 6.5 together with the mycelium and other solids in said liquor, and extracting the antibiotic from the thus separated solids.

13. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an acidified aqueous solution at a pH not exceeding 3 and separating the resulting aqueous antibiotic-containing solution from residual insoluble solids.

14. A method according to claim 13, which includes re-separating the antibiotic from said aqueous solution as a precipitate with calcium, barium, strontium or magnesium cations, at a pH above 6.5, extracting said product with an organic solvent, and recovering the tetracycline from the resulting extract.

15. A method according to claim 13, which includes extracting said aqueous solution with an organic solvent, separating the solvent layer from the aqueous layer, removing at least a part of the solvent layer to precipitate the antibiotic, and separating the latter.

16. A method according to claim 15, in which the extraction with an organic solvent is assisted by dissolving a salt in the aqueous layer.

17. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an organic solvent which is soluble in or miscible with water, preferably an alcohol such as butanol.

18. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an organic

ADMINISTRATION OF TETRACYCLINE

Animal protection tests using mice infected with *D. pneumoniae*, *K. pneumoniae* and *S. pyogenes* show that tetracycline when administered subcutaneously or orally, has activity comparable to that of oxytetracycline and chlortetracycline.

Tetracycline has been prepared in a purity in excess of 95% and with a negligible content of other antibiotic substances. An alternative form of the product comprises a significant content, not exceeding about one-half, of other compatible antibiotic substances either added to the preparation or formed concomitantly in the fermentation broth and co-crystallized in the recovery step, as when the above described butanol extraction or the extraction procedure of Example IV is employed. These products may be administered to humans and animals in the manner of known broad spectrum antibiotics, and in the conventional dosage forms appropriate to the desired route of administration.

What we claim is:—

1. A process of producing an antibiotic substance defined as tetracycline which comprises inoculating and aerobically fermenting an aqueous nutrient medium containing assimilable sources of carbon and nitrogen and mineral substances with a micro-organism of the genus *Streptomyces* which tends to produce chlortetracycline in the presence of chlorine ions, said method being characterized by controlling the chloride ion content of the nutrient medium so that the formation of the chlortetracycline is restricted and tetracycline is formed as the predominating antibiotic.

2. A method according to claim 1, in which the nutrient medium contains less than 1 ppm of available chloride ions.

3. A method according to either of claims 1 or 2, in which constituents of said medium normally containing available chloride ions have been treated with an ion exchange resin to remove said chloride ions.

4. A method according to any of claims 1 to 3, in which the nutrient medium is maintained at a pH from 3.5 to 8.5.

5. A method according to any of claims 1 to 4 in which the fermentation is carried out at a temperature of 25° C. to 35° C.

6. A method according to any of the preceding claims, in which the medium contains a source of calcium, strontium, barium, or magnesium ions whereby the antibiotic activity is precipitated as it is formed.

7. A method of recovering tetracycline from tetracycline-containing material which comprises forming a solution of said material, if not already in solution, and then adding a quaternary ammonium compound whereby the antibiotic is precipitated as a salt thereof.

8. A method according to claim 7, in which the quaternary ammonium compound is an

alkyl trimethyl ammonium chloride or a dialkyl dimethyl ammonium chloride in which each alkyl group contains from 8 to 18 carbon atoms.

9. A method according to either of claims 7 or 8, in which the precipitated quaternary ammonium salts are purified by solvent extraction.

10. A method according to claim 9, which comprises extracting the separated salts with chloroform in the presence of water, acidifying the chloroform extract to a pH of 1 to 2.5, adding water to form a chloroform phase and an aqueous phase containing the tetracycline, separating the aqueous phase, adjusting the pH of the aqueous phase to a pH of 3 to 7, and separating the resulting precipitated crystals of the antibiotic.

11. A method of recovering tetracycline from tetracycline-containing material which comprises forming a solution of said material, if not already in solution, effecting chromatographic adsorption of the antibiotic and eluting the latter.

12. A method of recovering tetracycline from tetracycline-containing material which comprises separating the antibiotic activity from the tetracycline-containing material as a precipitate with calcium, barium, strontium or magnesium cations, at a pH above 6.5 together with the mycelium and other solids in said liquor, and extracting the antibiotic from the thus separated solids.

13. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an acidified aqueous solution at a pH not exceeding 3 and separating the resulting aqueous antibiotic-containing solution from residual insoluble solids.

14. A method according to claim 13, which includes re-separating the antibiotic from said aqueous solution as a precipitate with calcium, barium, strontium or magnesium cations, at a pH above 6.5, extracting said product with an organic solvent, and recovering the tetracycline from the resulting extract.

15. A method according to claim 13, which includes extracting said aqueous solution with an organic solvent, separating the solvent layer from the aqueous layer, removing at least a part of the solvent layer to precipitate the antibiotic, and separating the latter.

16. A method according to claim 15, in which the extraction with an organic solvent is assisted by dissolving a salt in the aqueous layer.

17. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an organic solvent which is soluble in or miscible with water, preferably an alcohol such as butanol.

18. A method according to claim 12, in which the antibiotic is extracted from the solids by contacting the same with an organic



- solvent in the presence of water, at a pH below 3.
19. A method according to claim 18, in which an organic solvent is used which is soluble in or miscible with water, preferably an alcohol such as butanol.
20. A method of recovering tetracycline from tetracycline-containing material which comprises acidifying the same to a pH of less than 3, if necessary, to solubilize the resulting tetracycline, filtering, and recovering the antibiotic from the resulting aqueous solution.
21. A method according to claim 20, which includes acidifying the precipitated antibiotic to a pH of less than 3, filtering, adding sodium hydroxide to adjust the pH to between 8 and 10, extracting with an organic solvent, preferably an alcohol such as butanol, in the presence of a salt, separating the solvent layer from the aqueous layer, removing sufficient of the solvent under reduced pressure to cause the antibiotic to precipitate out, and filtering and recovering the thus precipitated antibiotic.
22. A method of producing an antibiotic defined as tetracycline substantially as hereinbefore described.

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ERRATA

SPECIFICATION No. 775,115

Page 4, Table II, line 6, *Micrococcus flavus*
PCI 16, column D. for "0.38" read
"0.39"

Page 9, line 82, for "0.3 g/10 ml" read
"0.3 g/100 ml"

Page 11, Table IV, for
"Chlortetracycline
mg/Kg."

read
"Chlortetracycline
mg/KG"

for
"Oxytetracycline
mg/Kg"

read
"Oxytetracycline
mg/KG"

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