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COMPLETE SPECIFICATION

Process for the Production of an Antibiotic Designated Kanamycin

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This invention relates to the preparation of a new antibiotic. More particularly it relates to processes for its production by fermentation, methods for its recovery and purification, as such or as its acid addition salts.

The new antibiotic produced according to this invention is called kanamycin and is effective in inhibiting the growth of Gram-positive, Gram-negative and acid fast bacteria such as *Mycobacterium tuberculosis*.

According to this invention kanamycin is prepared by cultivating a kanamycin-producing strain of *Streptomyces kanamyceticus* nov. spec. in an aqueous carbohydrate solution containing a nitrogenous nutrient under aerobic conditions until substantial antibacterial activity is imparted to said solution, whereafter the antibiotic is recovered and if necessary purified.

The micro-organism producing the antibiotic of the present invention was isolated from a sample of soils collected in Nagano Prefecture, Japan, and is a new species designated *Streptomyces kanamyceticus* of the genus *Streptomyces*.

A strain of *Streptomyces kanamyceticus* herein designated strain K2-J has the following characteristics:—

1. Microscopic observation: Substrate mycelium is about 1 μ in width and branched. The aerial mycelium developed from submerged mycelium branches and bears the sporophorous at the end. Spirals and whorls are not usually observed.

2. Glycerol-Czapek agar (27° C.): The

growth is colorless at first, lemon yellow later. The reverse side is hay-colored. Aerial mycelium is white to yellow, occasionally with a greenish or faint pinkish tinge. A faint brown soluble pigment occasionally.

3. Krainsky glucose asparagine agar (27° C.): Growth is colorless to yellow and the reverse side is white, faint pinkish white, yellow or hay-colored. Aerial mycelium is scant, usually developing from the center of the colony and is white, faint pinkish white, faint greenish yellow or yellow. No soluble pigment is usually found.

4. Calcium malate agar (27° C.): Same as on Krainsky glucose asparagine agar, but growth is slighter. Sometimes there is no growth.

5. Starch plate (27° C.): Almost same as Krainsky glucose asparagine agar, but growth is restricted and there is no aerial mycelium. Starch is hydrolyzed.

6. Potato plug (27° C.): Growth is wrinkled, granular surface, faint yellowish brown to yellow. Aerial mycelium is none or scant and white. There is no soluble pigment. Plug beneath the growth is occasionally dark.

7. Carrot plug (27° C.): Growth is usually scant. When grown it is almost the same as on potato plug.

8. Peptone water with 0.2% sodium nitrate (27° C.): Surface ring growth is colorless to white yellow, and white growth at bottom. White aerial mycelium occasionally occurs. Nitrite is formed from nitrate. There is no soluble pigment.

9. Peptone-meat extract agar (37° C.): Growth is wrinkled, white to yellow. There is no aerial mycelium or soluble pigment.

10. Blood agar (37° C.): Growth is wrinkled, reddish brown with grayish tinge. There is no aerial mycelium or soluble pigment.

11. Milk (37° C.): There is almost no change and growth is not usually observed at surface.

12. Loeffler coagulated serum (37° C.):

[Price 3s. 6d.]



occasionally

Growth is restricted, white to lemon yellow. There is no aerial mycelium or soluble pigment.

13. Egg medium (37° C.): Growth is wrinkled. There is no aerial mycelium.

14. Gelatin: Liquified; no soluble pigment.

15. Utilization of carbon sources: The following carbon sources were utilized on Czapek salt basal medium: arabinose, dextrin, fructose, galactose, glucose, glycerol, maltose, mannitol, mannose, raffinose, starch, sucrose, succinate. Carbon sources not utilized: inositol, inulin, lactose, rhamnose, sorbose, xylose, acetate. Carbon sources utilized poorly: esculin, salicin, sorbitol, citrate. Czapek salt basal medium has the following composition: NaNO_3 , 0.2%; K_2HPO_4 , 0.1%; MgSO_4 , 0.05%; KCl , 0.05%; FeSO_4 , 0.001%; distilled water, to 100%.

16. Production of the antibiotic, kanamycin.

The above characteristics are sufficient to distinguish the micro-organism from those hitherto described species of streptomycetes and to show that the strain K2-J belongs to a novel species.

The characteristics of this species may be summarized as follows: The growth of the colony is colorless to yellow with or without greenish or pinkish tinge. The reverse of the colony on synthetic agar is colorless, white, faint pinkish white, whitish yellow or hay-colored. The aerial mycelium is white to yellow and spirals and whorls are not formed. A faint brown soluble pigment is produced occasionally on a synthetic medium. Gelatin is liquified and starch is hydrolyzed.

A kanamycin-producing strain of *Streptomyces kanamyceticus* nov. spec. includes the typical strain No. K2-J described above and all natural and artificial variants or mutants thereof. A culture of this strain has been deposited with the American Type Culture Collection and assigned No. ATCC 12853.

PRODUCTION OF KANAMYCIN BY FERMENTATION.

Streptomyces kanamyceticus (K2-J) was first cultured in shake flasks in the following media: (a) 0.75% meat extract, 0.75% peptone, 0.3% NaCl , with 1.0% of starch, dextrin, maltose, glucose, lactose, sucrose, or glycerol; (b) 2.0% soybean meal, 0.05% KCl , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% NaCl , 0.2% NaNO_3 , with 1.0% of starch, dextrin, maltose, glucose, lactose, sucrose, or glycerol. The initial pH of all media was adjusted to 7.0. After 24–48 hours shaking in some cases the pH decreased to about 6.0–6.8, but from 72–120 hours the pH rose and became 7.5–8.6.

In the above medium (b) containing 2.0% starch, after 3 days of the shaking culture the pH rose to 8.2 and 250 mcg./ml. of kanamycin was produced. In the tank culture with the medium containing 2.0% starch, 0.5% glucose, 1.2% soybean meal, 0.05% KCl ,

0.05% MgSO_4 , 0.1% K_2PO_4 , 0.3% NaCl , 0.3% peptone and 0.3% CaCO_3 , the pH at the beginning was slightly lowered (6.6) and again increased and was 8.0 after 43 hours; 273 mcg./ml. of kanamycin was produced after 78 hours.

According to the present invention there is provided a process for the production of a new antibiotic, kanamycin, characterized by the fact that a kanamycin-producing strain of *Streptomyces kanamyceticus* nov. spec., of which the properties are described in the specification, is cultivated under aerobic conditions in an aqueous nutrient medium containing a source of carbon, nitrogen and inorganic salts, until substantial antibiotic activity is imparted to said medium, and the antibiotic is recovered from the fermentation broth.

In general terms, kanamycin is produced in media which contain organic nitrogen sources such as soybean meal, cotton seed meal, peanut meal, meat extract, peptone, corn steep liquor or yeast extract. The carbon sources which can be used include starch, dextrin, maltose, glucose, sucrose, lactose, and glycerol. The combination of starch and soybean meal was found to be one of the best and is preferred; the addition of corn steep liquor, peptone, yeast extract, or nitrate to this combination promoted the production of kanamycin. The addition of magnesium sulphate, manganese chloride, or zinc sulphate was promotive of good yields of kanamycin; the maximum yield was obtained when the pH of the culture broth was about 8. The temperature of the cultivation may be varied in a wide range, 25°–35° C., within which the organism may grow, but the temperature of 27°–32° C. is preferable. Generally the cultivation is continued until a substantial quantity of kanamycin is accumulated in the medium. It requires in the deep aerated submerged culture, in general, two to seven days with maximum production usually occurring at from 3 to 5 days. The kanamycin is found in the liquid portion of the fermentation broth.

EXPERIMENTAL METHODS

Shake culture: 150 ml. of the medium was placed in a flask of 500 ml. volume and sterilized. It was inoculated with the mycelia and spores of the kanamycin-producing organism on the agar medium or with mycelium obtained from a 48-hour shake culture and then shake-cultured on the reciprocating shaking machine (120 strokes per minute and 8 cm. amplitude) at 27°–29°.

Tank culture: A stainless steel fermenter of 400 l. volume was used. One hundred eighty liter or two hundred liter of the medium was placed therein and sterilized at 120° C. for 20 to 30 minutes. The aeration rate was 200 l. of air per minute and the r.p.m. of the stirrer was 200. Silicone, soybean oil or liquid paraffin was used as the antifoam.



EXTRACTION AND PURIFICATION OF KANAMYCIN.

In one preferred method of extraction, the kanamycin present in the whole fermentation both or in a clarified broth can be extracted with butanol in the presence of lauric acid or stearic acid. The antibiotic is transferred into butanol at pH 6—8, and from the solvent phase to the aqueous phase, at pH 2—4. In the absence of the lauric or stearic acid, kanamycin is not transferred significantly into butanol, ethyl acetate, butyl acetate or benzene. Thus extraction with these solvents can be used for the extraction of other co-existing substances and impurities.

In another method of recovering kanamycin from a fermentation broth, the broth is concentrated by vacuum evaporation or spray drying. The kanamycin can be extracted from the residue with water, methanol, ethanol or acetone; acidification with HCl improves the dissolving. The antibiotic can be adsorbed by activated carbon from neutral or alkaline aqueous solution and subsequently eluted into dry or aqueous alcohol or aqueous acetone adjusted to pH 2.0 with hydrochloric acid. Kanamycin is also adsorbed at a pH about 6.0 to 9.0 by cation exchange resins preferably of the carboxylic acid type, such a copolymer of methacrylic acid and divinylbenzene. When adsorbed on a cation exchange resin the antibiotic can be eluted with aqueous hydrochloric acid, aqueous sulphuric acid, or acidified aqueous organic solvents (for instance, 1NHCl—10% aqueous acetone). Kanamycin can also be eluted by ammonium hydroxide.

The eluate thus obtained can be concentrated by vacuum evaporation or by freeze-drying, and the kanamycin subsequently precipitated from the concentrated solution so formed by adding a solvent in which kanamycin or its salt is substantially insoluble.

In still another method of purification one can use column chromatography with alumina or active carbon. For instance, an aqueous solution of kanamycin (pH 8.0) obtained by the repetition of the cation exchange resin process can be passed through a column containing active carbon and, after washing the column with distilled water, elution is made with 0.5 NH_4SO_4 . The most active fraction of the eluate is added to methanol and crystalline kanamycin sulphate precipitated.

NATURE OF KANAMYCIN.

Kanamycin is isolated as its hydrochloride or sulphate and the free base of kanamycin is obtained from these salts, for instance, removing the sulphuric ion by adding barium hydroxide to a kanamycin sulphate solution.

Kanamycin hydrochloride is freely soluble in water, methanol, slightly soluble in ethanol and insoluble in acetone, ethyl acetate, butyl acetate, benzene, ether or petroleum ether.

Kanamycin sulphate is soluble in water and

insoluble in methanol, ethanol, acetone, ethyl acetate, and benzene.

The kanamycin base is soluble in water and substantially insoluble in n-butanol, ethyl acetate, butyl acetate, ether, chloroform, and benzene.

Kanamycin when dissolved in pyridine and tested, gives positive ninhydrin reaction. Tollens, Sakaguchi, Fehling, Molisch and glucosamine (Elson-Morgan) tests are negative; even after hydrolysis, the glucosamine test is negative. When the paper strip chromatography was carried out (with Toyo filter paper No. 50 and 0.2% p-toluenesulphonic acid-butanol saturated with water) the R_f value of kanamycin was 0.21—0.26.

Kanamycin exhibits optical activity. When a 1.0% aqueous solution of purified kanamycin hydrochloride was tested, $[\alpha]_D^{17}$ was $+103^\circ$ and when a 1.0% aqueous solution of kanamycin free base was tested, $[\alpha]_D^{17}$ was $+112^\circ$, and when a 1.0% aqueous solution of crystalline kanamycin hemisulphate was tested, $[\alpha]_D^{25} +121^\circ$. Analytical data for free base: Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}$: C, 40.48; H, 7.04; N, 10.12; O, 42.37; found: C, 43.17; H, 6.95; N, 9.82. Analytical data for the hydrochloride: Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}\cdot 3\text{HCl}$: C, 32.04; H, 6.15; N, 8.01; Cl, 20.27; O, 33.54; found: C, 31.31; H, 6.07; N, 8.21; Cl, 18.03. Analytical data for kanamycin sulphate (amorphous): Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}\cdot 3/2\text{H}_2\text{SO}_4$: C, 29.89; H, 5.73; N, 7.47; S, 8.57; O, 48.35; found: C, 30.20; H, 5.88; N, 7.43. Analytical data for N-acetyl kanamycin: Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}\cdot 3\text{CH}_3\text{CO}$: C, 44.36; H, 6.51; N, 7.76; O, 41.36; found: C, 44.39; H, 6.01; N, 7.49. Analytical data for kanamycin reineckate: Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}\cdot 3(\text{C}_6\text{H}_4\text{N}_2\text{S}_2\text{Cr})$: C, 22.68; H, 3.88; N, 21.37; S, 27.95; Cr, 11.33; O, 12.78; found: C, 21.44; H, 4.31; N, 19.93; Cr, 11.40 (from ash content). Analytical data for crystalline kanamycin hemisulphate: Calculated for $\text{C}_{14}\text{H}_{29}\text{N}_5\text{O}_{11}\cdot 1/2\text{S}_2\text{O}_8$: C, 36.21; H, 6.51; N, 9.05; S, 3.44; O, 44.79; found: C, 35.97; H, 6.51; N, 9.13; S, 3.47.

Kanamycin base exhibits no absorption of ultraviolet light from 220 $\text{m}\mu$ to 400 $\text{m}\mu$.

In the accompanying drawing, the figure shows the infra-red absorption spectrum of crystalline kanamycin hemi-sulphate pelleted in potassium bromide.

It is evident from the drawing that the new antibiotic has a novel and characteristic configuration. The characteristic absorption bands exhibited in the infra-red region of the spectrum are at the following wave lengths expressed in microns: 2.85, 3.03, 3.20, 3.30, 3.43, 3.63, 3.70, 3.83, 4.70 (broad), 6.05, 6.17, 6.27, 6.60, 6.90, 7.15, 7.35, 7.52, 7.60, 7.95, 8.18, 8.76, 8.95, 9.08, 9.40, 9.70, 10.20, 10.48, 10.75, 11.13, 11.50, 11.90, 12.35, 12.80, and 13.15.

Among known antibiotics Neomycin B and

C, neamine and catenulin (see Waksman and Lechevalier, "Actinomycetes and Their Antibiotics", 1953, p. 199) have some characteristics in common with kanamycin. But the following characteristics differentiate kanamycin definitely from these antibiotics. The glucosamine test for neomycin shown in the book "Assay Methods of Antibiotics" by D. C. Grove and W. A. Randall, 146 pages, (medical Encyclopedia Inc. New York) is negative for kanamycin. This reaction is positive for the neomycins and catenulin. Paper chromatography with a 2.0% p-toluenesulphonic acid-butanol saturated with water differentiates kanamycin from neamine. When the Toyo filter paper No. 50 was used, Rf value of kanamycin was 0.21—0.26, and that of neamine was 0.75—0.85. By the broth dilution method,

kanamycin inhibited the growth of *Klebsiella pneumoniae* at 3 mcg./ml., neamine at 25 mcg./ml., neomycin B at 3 mcg./ml., catenulin at 1.5 mcg./ml. By the cylinder plate method with *B. subtilis*, for the exhibition of 20 mm. inhibition diameter, 30 mcg./ml. of kanamycin, 25 mcg./ml. of neamine, 170 mcg./ml. of neomycin B, 250 mcg./ml. of neomycin C, or 18 mcg./ml. of catenulin was required. These characteristics as well as the property of low toxicity and empirical formulae confirmed the fact that kanamycin is different from the neomycins, neamine and catenulin and is a new antibiotic.

ACTIVITY OF KANAMYCIN.

Kanamycin, when tested by the agar dilution method, showed the following antibacterial spectrum:—

Minimum concentration for complete inhibition in mcg./ml.

Micro-organisms	
40	<i>S. lutea</i> PCI-1001 - - - -
	<i>M. pyogenes</i> var. aureus 209-P - -
	<i>M. pyogenes</i> var. aureus Terajima -
	<i>Micrococcus flavus</i> - - - -
	<i>B. subtilis</i> PCI-219 - - - -
	<i>B. subtilis</i> NRRL-558 - - - -
45	<i>B. subtilis</i> Tracy - - - -
	<i>E. coli</i> - - - -
	<i>S. paratyphi</i> A - - - -
	<i>S. dysenteriae</i> - - - -
	<i>Proteus vulgaris</i> - - - -
50	<i>Mycobacterium</i> 607 - - - -
	<i>Mycobacterium phlei</i> - - - -
	<i>Saccharomyces sake</i> - - - -
	<i>Saccharomyces</i> - - - -
	<i>Candida albicans</i> - - - -
55	<i>Aspergillus niger</i> - - - -

1.8

2.2

0.4

9.0

4.5

4.5

9.0

2.2

3.1

1.6

6.2

3.1

2.2

300

300

300

300

In Kirchners medium it inhibited *Mycobacterium tuberculosis* H₃ strain at 2 mcg./ml. The streptomycin resistant *E. coli* and streptomycin-fast *Mycobacterium tuberculosis* hominis was sensitive to kanamycin.

Mice tolerated the intravenous injection of 150 mk./kg. LD₅₀'s i.v. in mice, rats and rabbits were 150—250 mg./kg. Mice tolerated daily subcutaneous injection of 200 mg./kg. for 30 days. No side reactions were observed on daily injection of 100—200 mg./kg. of kanamycin in dogs for 30 days. Intraperitoneal injection of more than 50 mcg./mouse every four hours protected mice infected with 10000 MLD of *Diplococcus pneumoniae* or 1000 MLD of *Salmonella typhosa*. Daily subcutaneous injection of 100 mg./kg. of kanamycin showed the inhibitory effect on mice

infected with *Mycobacterium tuberculosis* (Ravenel strain).

Following is a description by way of example of methods of carrying the invention into effect.

EXAMPLE 1.

Glucose, glycerol, lactose, maltose, sucrose, starch or dextrin was added at 2.0% to the basal medium containing 0.75% meat extract, 0.75% peptone, and 0.3% sodium chloride and sterilized and the pH after the sterilization was adjusted to 7.0. The medium was inoculated with one loopful of a glycerol Czapek agar slant of *Streptomyces Kanamyceticus* and shake-cultured at 27°—29° C. The production of kanamycin was determined by the cylinder plate method with *Mycobacterium* 607, and the following results were observed:—

20

25

30

35

75

80

85

90

Carbon source	Maximum production of kanamycin	Final pH and number of days of shaking culture to attain maximum production	
Starch	250 mc./ml.	7.8	5 days
Dextrin	200	8.0	5
Maltose	130	7.8	4
Glucose	200	8.2	4
Lactose	100	8.6	4
Sucrose	100	8.4	4
Glycerol	100	8.0	4

When a carbohydrate as indicated was added to the basal medium containing 1.0% soybean meal, 0.05% KCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.3% NaCl, the following results were observed:—

Carbon source	Maximum production of kanamycin	Final pH and number of days of shaking culture to attain maximum production	
Starch	250 mcg./ml.	7.6	4 days
Dextrin	250	8.0	4
Maltose	130	7.8	4
Glucose	100	8.2	4
Sucrose	150	8.4	4
Lactose	100	8.4	3
Glycerol	70	8.2	4

EXAMPLE 2.

The medium containing 2.0% starch, 1.2% soybean meal, 0.05% KCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% K_2HPO_4 , 0.3% NaCl, 0.3% peptone and 0.5% CaCO_3 (the initial pH was 7.0), was inoculated with the growth of a selected subculture of *S. kanamyceticus* (strain K2-J) on glycerol Czapek agar and cultured. After days of shake culture the pH was 8.2 and assays by the cylinder plate method with both *B. subtilis* and *Mycobacterium* 607 indicated the production of 550 mcg./ml. of kanamycin.

EXAMPLE 3.

The medium (180 l.) containing 2.0%

starch, 1.0% glucose, 1.2% soybean meal, 0.3% NaCl, 0.05% KCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% K_2HPO_4 , 0.2% CaCO_3 , 0.3% NaNO_3 , 0.002% $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.002% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was placed in a fermenter of 400 l. volume, adjusted to pH 7.4, sterilized for 30 minutes at 120° C., inoculated with 1000 ml. of 40 hour shake-cultured broth of *S. kanamyceticus* (a selected subculture of K2-J strain) and tank-cultured at 27°—29° C. As anti-foam, soybean oil (0.04%) and silicone (0.04%) were added. The results were as follows:—

Hrs.	pH	Kanamycin mcg./ml.	Reducing sugar % in liquid	NH_3 -N mg. % in liquid
0	6.6	—	2.20	2.5
24	6.9	—	1.50	10.7
36	7.6	150	0.4	1.2
48	7.6	200	0.3	1.2
60	8.0	220	0.3	6.0
72	8.2	210	0.25	12.0

EXAMPLE 4.

The medium (200 l.) containing 2.0% starch, 1.0% soybean meal, 0.05% KCl, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% NaCl, 0.2% NaNO_3 was placed in the 400 liter fermenter, the pH was adjusted to 7.5, and the medium was then sterilized (pH after the sterilization was 7.0), and inoculated and cultured as in

case of the Example 3. The broth after 48 hours was found to contain 250 mcg./ml. of kanamycin. Thereafter, the concentration of kanamycin in the broth was not markedly changed. After 65 hours of the fermentation, the broth was filtered. Thus 160 l. of filtered broth was obtained. The filtered broth contained 150 mcg./ml. of kanamycin and the pH

was 8.2. The filtrate was passed through a cation exchange resin column. The column was 15 cm. in diameter and contained 6 l. of "Amberlite IRC-50" (Registered Trade Mark) in the sodium form (i.e. regenerated with sodium hydroxide) at pH 8.0. "Amberlite IRC-50" is a commercially available cation exchange resin of the carboxylic type; it is a copolymer of methacrylic acid and divinyl benzene. The filtrate was passed through the column at the rate of 16 l./hour. In the effluent no antibacterial activity against *Mycobacterium* 607 was detected by the cylinder plate method. The column was then washed with about 10 l. of distilled water which was passed through the column at the same speed as the broth filtrate. Thereafter, 1NHCl was passed through the column at the rate of 0.8 l./hour. The eluate was collected in 2 l. portions (cuts). After the 7th cut the eluates became acidic and the kanamycin was found in the 7th-10th cuts inclusive. The cuts containing kanamycin were combined and the combined solution (8 l. in volume) was adjusted to pH 6.0 with 10% NaOH, and concentrated (by distillation under vacuum at about 50° C.) to 3000 ml. The concentrated solution was freeze-dried. The brownish white powder of 500 g. thus obtained contained 8 g. of kanamycin. It was dissolved in 2 l. of methanol, and, after the insoluble part was removed, acetone (20 l.) was added and 80 g. of the brownish white powder was obtained. This powder contained 8 g. of kanamycin. Twenty grams of this powder were dissolved in 40 ml. of distilled water and saturated aqueous ammonium reineckate solution was added. The light pinkish precipitate which first appeared and which weighed 50 mg. was removed, and more ammonium reineckate solution was added until no more precipitate formed. This pinkish precipitate was collected and recrystallized from distilled water, and crystalline pink kanamycin reineckate (300 mg.) was obtained. It darkened at 191°-193° C. and decomposed at 211°-213° C. The light pinkish precipitate which first appeared also contained kanamycin.

EXAMPLE 5.

S. kanamyceticus (a selected subculture of K2-J strain) was inoculated to 200 l. medium placed in a 400 l. stainless steel fermenter and fermented. The medium contained starch 2.0% glucose 1.0% soybean meal 1.2%, NaCl 0.3%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, K_2HPO_4 0.1%, $CaCO_3$ 0.2% and peptone 0.3%. The pH of the medium after sterilization was 7.0. Soybean oil was added to the medium at 0.1% as an antifoam. After sixty-five hours of aerated deep culture, the broth contained 220 mcg./ml. of kanamycin. The pH was 8.0. It was filtered and 170 l. of the filtrate was obtained (210 mcg./ml.). It was passed through the cation exchange resin tower. The resin tower contained 6 l. of Amberlite IRC-50 resin in

the sodium cycle at pH 8.0 and was 15 cm. in diameter. The rate of flow was 30 l./hour, that is, 1/12 liter of resin volume per minute. Thereafter, 30 l. water was passed through the resin tower at the rate of 30 l./hour. Kanamycin adsorbed by the resin was then eluted with 1NHCl, the flow rate being 3.0 l./hour. The first eluate (6.5 l.) contained no activity; the following eluate contained kanamycin. Ninety per cent of the adsorbed kanamycin appeared in the eluate, the pH of which was higher than 2.0. The active eluate (16.1) was adjusted to pH 7.5 with 10% NaOH. It was then diluted to 80 l. with water. The yield here from the broth filtrate was 83%. The diluted solution was passed again through a resin column. The column contained from 2 l. of "Amberlite IRC-50" resin in the sodium cycle at pH 7.5. The diameter of the column was 5 cm. The rate of the flow was 10 l./hour. The column was washed with 20 l. of water at a rate of flow of 10 l./hour. The adsorbed kanamycin was then eluted with 1NHCl. The rate of flow was 3.0 l./hour. As in the previous elution kanamycin appeared in the eluate, the pH of which was higher than 2.0. The active eluate (4.5 l.) was adjusted to pH 6.0 by the addition of the anion exchange resin, "Amberlite IR-4B", in the hydroxyl form. "Amberlite IR-4B" is a commercially available weakly basic anionic exchange resin. It was evaporated under vacuum at about 40° C. to 500 ml. To this concentrated solution 5 liters of methanol was added and the insoluble part was removed. The filtrate was evaporated under vacuum at about 40° C. to 250 ml. and to the concentrated solution 2.5 liters of acetone was added and 65 g. kanamycin was obtained as a light brownish powder. The potency of this powder was 350 mcg./mg.

EXAMPLE 6.

The brownish white kanamycin (5 g.) obtained in Example 5 was dissolved in 50 ml. of 60% aqueous methanol, insoluble material was removed and to the filtrate 40 ml. of 60% aqueous methanol containing 2000 mg. of ammonium sulphate was added, and the precipitated kanamycin sulphate was collected, washed with 50 ml. of 80% aqueous methanol, and dried. Thus 4.5 g. of kanamycin sulphate was obtained as a light brownish powder. The potency was 370 mcg./mg.

EXAMPLE 7.

Ten grams of solid kanamycin hydrochloride, the potency of which was 535 mcg./mg., was dissolved in 100 ml. of 90% aqueous methanol and passed through an alumina column. The column contained 140 g. of alumina treated with sulphuric acid and was 1.5 cm. in diameter. It was developed with methanol. The first eluate of 200 ml. did not contain kanamycin. The second eluate (128 ml.) showed antibacterial activity and was eva-

porated under vacuum to obtain 4.681 g. of kanamycin hydrochloride as a white powder. The potency of the powder was 560 mcg./mg. By the same procedure, from the third eluate (115 ml.) 2.205 g. of solid white kanamycin hydrochloride was obtained. The potency was 615 mcg./mg. By this procedure, the yellow pigment was adsorbed by the column and the kanamycin hydrochloride was obtained as a colorless powder. The yellow pigment adsorbed by the column was eluted by the further elution with 50% aqueous methanol and distilled water.

EXAMPLE 8.

Four grams of solid kanamycin hydrochloride, the potency of which was 500 mcg./mg., was dissolved in 200 ml. of methanol and after evaporation to 80 ml. was passed through a column containing 5 g. of active carbon and 25 g. of cellulose powder. The column was developed with methanol. The first eluate (50 ml.) did not contain kanamycin. The second eluate (60 ml.) was evaporated to obtain 364 mg. of kanamycin hydrochloride. The potency of this powder was 720 mcg./mg. The third eluate (46 ml.) gave 381 mg. of kanamycin hydrochloride powder. The potency of this powder was 616 mcg./mg. From the fourth eluate (55 ml.) 1873 mg. of white kanamycin hydrochloride was obtained having a potency of 483 mcg./mg. The fifth eluate (55 ml.) gave 956.6 mg. as a white powder having a potency of 410 mcg./mg. All solids here obtained were colorless.

EXAMPLE 9.

S. kanamyceticus was shake-cultured in a medium containing 1.0% starch, 1.5% soybean meal and 0.3% NaCl (the initial pH was 7.0) at 28° C. for four days. The culture broths in 25 flasks were combined and filtered. The pH of the filtrate was 8.2 and the filtrate contained 400 mcg./ml. kanamycin. The filtrate (3100 ml.) was passed through "Amberlite IRC-50" resin column. The column contained 100 ml. of the resin in the sodium cycle at pH 8.0. After absorption, the column was washed with 500 ml. of water, and the adsorbed kanamycin was eluted with 1N HCl, cutting the effluent each 20 ml. The activity was included in the fourth to ninth cuts; these cuts were combined and the pH was adjusted to 6.0. This solution was concentrated by vacuum evaporation to 12 ml. and insoluble impurities were removed. To this solution saturated aqueous ammonium reineckate solution was added. The precipitate which first appeared was light pinkish colored, weighed 50 mg., contained kanamycin and was separated. To the supernatant further ammonium reineckate solution was added until no more precipitates formed. The pink precipitate was separated and dried to give 544 mg. kanamycin reineckate; about 30% of the activity was left in the supernatant. This precipitate was added to 12 ml. of distilled water, warmed to 60° C. and filtered.

The filtrate was cooled and kanamycin reineckate crystals were separated. To the mother liquor kanamycin reineckate which had not been redissolved was added and after again heating to 60° C. and cooling the second crop of crystalline kanamycin reineckate was separated. This process was further repeated three times. Thus, 300 mg. of crystalline kanamycin reineckate was isolated. This crystalline reineckate (140 mgm.) was added to 105 ml. of distilled water and 2.5N HCl in pyridine was added drop by drop until no more precipitate formed. The precipitate was removed by centrifuge, freeze-dried and washed with acetone. This kanamycin hydrochloride (60 mg.) was obtained as a hygroscopic white solid.

EXAMPLE 10.

Kanamycin hydrochloride can be prepared from the kanamycin base obtained by the process of Example 5 by dissolving the base in the minimum amount of water and adding 2.5N HCl in pyridine until precipitation is complete. The kanamycin hydrochloride can be separated by centrifugation, freeze-dried and washed with acetone. The grams of kanamycin hydrochloride (450 mcg./mg.) obtained in this way was dissolved in one liter of distilled water and the pH was adjusted to 6.4. It was added to a column of 75 ml. of "Amberlite IRC-50" resin in the sodium form at pH 6.4 at a flow rate of 10 ml./minute. After the column was washed with 50 ml. water, the adsorbed kanamycin was eluted by 5% NH₄OH. The first eluate (80 ml.) had no activity and the further eluate (130 ml.) contained kanamycin. The pH of this eluate was higher than 10.0. It was evaporated under vacuum at about 40° C. to 22 ml. The yield in this concentrated solution was 97.8%. This concentrated solution was passed through a carbon column containing 20 g. of active carbon of 100-250 mesh. The diameter of the column was 2 cm. Thereafter the column was washed with 160 ml. of distilled water and chromatographically developed with 0.5N H₂SO₄. The effluent was cut in about 20 ml. portions. The kanamycin sulphate appeared from the 5th cut. The 1st-4th cuts were pH 6.2-6.4 and did not contain kanamycin. Almost all of the kanamycin was found in the 5th-8th cuts as follows: 5th cut, pH 8.2, 22.0 ml., 16.9 mg./ml.; 6th cut, pH 8.6, 23.0 ml., 65.3 mg./ml.; 7th cut 8.6, 20.0 ml., 55.0 mg./ml.; 8th cut pH 4.6, 22.0 ml., 47.5 mg./ml.; 9th cut, pH, 1.0, 22.0 ml., 4.2 mg./ml. To the 6th cut 23.0 ml. of methanol was added and crystalline kanamycin sulphate was precipitated, collected and dried. Thus 1.39 g. of crystalline kanamycin sulphate was obtained. 1.2 g. of this crystalline sulfate was dissolved in 8 ml. of water and warmed to 45° C. and 6.5 ml. of methanol was added with stirring and cooling. Then, kanamycin sulphate (1.07 g.) was precipitated and was obtained in crystalline form. Microscopically it was color-



less plate crystals. It did not melt below 250° C.

Analytical results: C, 35.97, 35.89; H, 6.51, 6.56; N, 9.13, 8.92; S, 3.47; $[\alpha]_D^{25} = +121^\circ$.

- 5 Kanamycin and its therapeutically acceptable salts such as the hydrochloride and sulphate may be used medicinally in admixture with a pharmaceutical carrier, as such, or with the further addition of one or more medicaments
- 10 such as antihistamines, sulpha drugs, stimulants, local anaesthetics, analgesics, laxatives, sedatives, penicillin salts and other antibiotic agents such as streptomycin or one of the tetracycline antibiotics, vitamins, hormones, and
- 15 antifungal agents.

WHAT WE CLAIM IS:—

1. A process for the production of a new antibiotic, kanamycin, characterized by the fact that a kanamycin-producing strain of *Streptomyces kanamyceticus nov. spec.*, of which the properties are described in the specification, is
- 20 cultivated under aerobic conditions in an aqueous nutrient medium containing a source of carbon, nitrogen and inorganic salts, until substantial antibiotic activity is imparted to said medium and the antibiotic is recovered from the fermentation broth.

2. A process according to Claim 1, wherein the cultivation is carried out under submerged
- 30 conditions at a temperature in the range from 27° to 32° C. for a period of 3 to 5 days.

3. A process according to Claims 1—2, wherein the nutrient medium contains the combination of starch and soybean meal.

- 35 4. A process according to Claim 3, wherein the amounts of starch and soybean meal in the nutrient medium are 2.0% and 1.2% respectively.

5. A process according to Claims 1—4 wherein the antibiotic is recovered from the fermentation broth by extraction with butanol
- 40 in the presence of lauric or stearic acid.

6. A process according to Claims 1—4, wherein the antibiotic is recovered from the fermentation broth by way of adsorption on a
- 45 cation exchange resin.

7. A process according to Claim 6, wherein

the cation exchange resin is of the carboxylic acid type.

8. A process according to Claims 6—7, wherein the cation exchange resin is a copolymer of methacrylic acid and divinyl benzene.

9. An antibiotic substance herein designated kanamycin effective in inhibiting the growth of Gram-positive bacteria, Gram-negative bacteria and mycobacteria consisting of a basic compound capable of forming salts with acids, soluble in water and substantially insoluble in n-butanol, ethyl acetate, butyl acetate, ether, chloroform and benzene, exhibiting no absorption of ultraviolet light from 220 mμ to 400 mμ, giving a positive reaction to ninhydrin reagent dissolved in pyridine and negative Tollens, Sakaguchi, Fehling, and Elson-Morgan reactions and, in the form of its purified hemi-sulphate, containing 35.9% carbon, 6.5% hydrogen, 9.1% nitrogen and 3.5% sulphur by analysis and 45.0% oxygen by difference, exhibiting $[\alpha]_D^{25}$ of $+121^\circ$ (1% solution in water) and exhibiting characteristic absorption bands in the infra-red region of the spectrum when pelleted in potassium bromide at the following wave lengths expressed in microns: 2.85, 3.03, 3.20, 3.30, 3.43, 3.63, 3.70, 3.83, 4.70 (broad) 6.05, 6.17, 6.27, 6.60, 6.69, 7.15, 7.35, 7.52, 7.60, 7.95, 8.18, 8.76, 8.95, 9.08, 9.40, 9.70, 10.20, 10.48, 10.75, 11.13, 11.50, 11.90, 12.35, 12.80, and 13.15.

10. An acid addition salt of the basic antibiotic claimed in Claim 9.

11. A hydrochloride of the basic antibiotic claimed in Claim 9.

12. A sulphate of the basic antibiotic claimed in Claim 9.

13. A reineckate of the basic antibiotic claimed in Claim 9.

14. A process for the production of a new antibiotic herein designated kanamycin substantially as described with reference to any one of the specific examples hereinbefore set forth.

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Chartered Patent Agents.



carried

butanol

AW



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10 such as antihistamines, sulpha drugs, stimulants, local anaesthetics, analgesics, laxatives, sedatives, penicillin salts and other antibiotic agents such as streptomycin or one of the tetracycline antibiotics, vitamins, hormones, and
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2. A process according to Claim 1, wherein the cultivation is carried out under submerged conditions at a temperature in the range from 27° to 32° C. for a period of 3 to 5 days.

3. A process according to Claims 1—2, wherein the nutrient medium contains the combination of starch and soybean meal.

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- 40 5. A process according to Claims 1—4 wherein the antibiotic is recovered from the fermentation broth by extraction with butanol in the presence of lauric or stearic acid.

- 45 6. A process according to Claims 1—4, wherein the antibiotic is recovered from the fermentation broth by way of adsorption on a cation exchange resin.

7. A process according to Claim 6, wherein

the cation exchange resin is of the carboxylic acid type.

8. A process according to Claims 6—7, wherein the cation exchange resin is a copolymer of methacrylic acid and divinyl benzene.

9. An antibiotic substance herein designated kanamycin effective in inhibiting the growth of Gram-positive bacteria, Gram-negative bacteria and mycobacteria consisting of a basic compound capable of forming salts with acids, soluble in water and substantially insoluble in n-butanol, ethyl acetate, butyl acetate, ether, chloroform and benzene, exhibiting no absorption of ultraviolet light from 220 mμ to 400 mμ, giving a positive reaction to ninhydrin reagent dissolved in pyridine and negative Tollens, Sakaguchi, Fehling, and Elson-Morgan reactions and, in the form of its purified hemi-sulphate, containing 35.9% carbon, 6.5% hydrogen, 9.1% nitrogen and 3.5% sulphur by analysis and 45.0% oxygen by difference, exhibiting $[\alpha]_D^{25}$ of $+121^\circ$ (1% solution in water) and exhibiting characteristic absorption bands in the infra-red region of the spectrum when pelleted in potassium bromide at the following wave lengths expressed in microns: 2.85, 3.03, 3.20, 3.30, 3.43, 3.63, 3.70, 3.83, 4.70 (broad) 6.05, 6.17, 6.27, 6.60, 6.69, 7.15, 7.35, 7.52, 7.60, 7.95, 8.18, 8.76, 8.95, 9.08, 9.40, 9.70, 10.20, 10.48, 10.75, 11.13, 11.50, 11.90, 12.35, 12.80, and 13.15.

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