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SUBJECT OF INVESTIGATION

STUDIES ON THE MODE
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ANTIBACTERIAL DRUGS.

~~RESPONSIBLE INVESTIGATOR~~

~~Dr.~~ Katsuhiko Tago.

~~Assist. Chief. Tuberculosis Section.~~

Kitasato Inst~~itute~~

~~Tokyo,~~ (Japan).

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1. Preparation of C^{14}-leucomycin by fermentation using radio-active precursors.

a. Materials and Methods.

(1) Strain used: Streptomyces kitasatoensis was used in these studies. The strain was transfered to slants of either leucomycin medium or starch synthetic medium to which 2 % agar was added. The slant cultures were incubated 3 to 7 days. The spores were gently scraped from the culture surface to form a spore inoculum.

(2) Medium used: The following two media were used throughout the studies.

(a) Leucomycin medium.

Glucose	4.0g
NaCl	0.5g
K_2HPO_4	0.1g
$(NH_3)SO_4$	0.3g
C.S.L.	0.5g
Urea	0.05g
Soi-bean powder	2.0g
Yeast extract	0.5g
$CaCO_3$	0.3g
Aq. dest.	100 ml.

(b) Starch-synthetic medium.

Soluble starch	0.2g
K_2HPO_4	0.05g
$MgSO_4$	0.02g
$CaCl_2$	0.02g
$NaNO_3$	0.005g
Asparagin	0.005g
$Fe_2(SO_4)_3$	0.001g
Aq. dest.	100 ml.

Adjust pH 7.4. Agar is added 2.0% in solid medium.

(3) Method of fermentation: 100 ml. of starch medium was distributed to 250 ml. Er-flasks and inoculated 1.0 ml. of 48 hrs. culture of S. kitasatoensis in leucomycin media. Labeled precursors were added to the medium either as solid prior to sterilization or as awueous colution which had been passed through an ultrafine bacteriological filter and added after sterilization. The fermentation was carried out at 30 C on a shaker.

(4) Isolation of leucomycin: After filtration of mycelium, the culture filtrate was extracted twice with 50 ml. of ether which was evaporated in vacuo. Dried leucomycin was solved with 1.0 ml. of ether and diluted with adequete anount of aq. dest.

(5) Estimation of radio-activity: 1.0 ml. of solution of leucomycin was distributed to alminum cup and vacum-dried. The 14-C content of dried preparation was estimated by use of 'Riken' radiation counter model RSC-SB.

b. Result

(1) Fig. 1 showes the relationship between pH value of culture filtrates and incubation period. All media showed the reversal of pH value on the fourth day of the incubation. Among the media tested, leucomycin medium showed the most significant reversal and starch media inoculated spores of S. kitasatoensis slightest reversal. Production of leucomycin in media was paralleled to the reversal of media. Contents of leucomycin in leucmycin-medium was 40 mcg. per ml. and the lowest starch medium inoculated spores was 5 mcg per ml. The optimal concentration of soluble starch in synthetic medium was 0.2 g per liter.

(2) Twenty hours after inoculation of S. Kitasatoensis in 100 ml. of starch-synthetic medium, 0.1 mc. of C^{14}-sodium acetate or C^{14}-Starch-U were added as the precursor. Three days after addition of them, radioactive leucomycin was extracted from the culture filtrate. C^{14}-acetate-leucomycin showed very high specific activity, 208,000 cpm per u mol, whereas C^{14}-starch leucomycin was only 260 cpm per u mol.

2. Fractionation of Staphylococcal cells treated with radioactive leucomycin.

a. Materials and Methods.

(1) Cold TCA fraction: All low-molecular weight compounds soluble in 5 % (w/v) teichloroacetic acid are contained. The organism was suspended in 2 ml. cold water, added 0.5 ml. cold 25 % (w/v) TCA; after 10 min., centrifuged at 4,000 g for 5 min., and decanted extract.

(2) Aqueous ethanol-soluble fraction: Ethanol-soluble 'protein' and lipid are contained. The residue was suspended in 2.5 ml. 75% (w/v) ethanol in water; after 10 min. at room temperature centrifuged (4,000 g 10 min.) and decanted extract.

(3) Hot TCA fraction: Breakdown products of nucleic acid and teichoic acid. The residue was suspended in 2,5 ml. 5% (w/v) TCA, heated 6 min. at 90 C, cooled, centrifuged (4,000g, 10 min.) and decanted extract.

(4) Trypsin-solubilized: Trypsin-degraded proteins. The residue was suspended in 0.95 ml. 0.05 N-NH_4HCO_3 containing 0.005 N-NH_4OH:0.05 ml. of solution containing 1 mg. crystalline trypsin per ml. was added. Incubated 2 hrs. at 37 C or until digestion was complete. Centrifuged (4,000 g, 10 min.) and decanted extract.

(5) Residue: Mucopeptide of wall. The residue was suspended in 1.0 ml. of water.

b. Results.

Staphylococcal cells treated with C^{14}-leucomycin were fractionated by a modified method of Park et al. and radio activity of each fraction was estimated. As shown in Table 2, the high radio activity were revealed in the trypsin digested protein fraction and cell wall mucopeptide fraction, whereas low radio activity were found from ethanol soluble lipid fraction and nucleic acid fraction.

Table 1. Fractionation of *Staphylococcus aureus* treated with C^{14}-leucomycin.

Fraction	Contents of fraction	cpm
1. Cold TCA	All low-molecular weight compounds soluble in 5% (w/v) TCA	35.0
2. Aqueous ethanol soluble	Ethanol-soluble 'protein' and lipid	11.5
3. Hot TCA	Breakdown products of nucleic acid and teichoric acid.	0
4. Trypsin-solubilized	Trypsin-degraded protein	52.5
5. Residue	Mucopeptide of wall	70.0

3. Preparation of ribosoms from staphylococcal cells treated with radioactive leucomycin.

a. Materials and Methods

Bacterial extracts were prepared by grinding washed whole cells with alumina. The alumina and cells (3:1) were homogeneously mixed in an ice cold mortar. To the white paste-like material a volume of extracting solvent was added equal to the wet weight of cells. The solvent contained: Tris buffer (0.1M pH 7.2); $MgCl_2$ (7×10^{-3}M); and KCl (6×10^{-2}M). The mixture of solvent, cells and alumina was vigorously ground for 5 min. Precipitable material was removed by centrifugation (10 min, 10,000g). The precipitate was re-extracted two more times as before, except 2 volumes of extracting solvent were used each times; the supernatants of all centrifugations were then combined and recentrifuged (120 min. 105,000g). The radiation count of the precipitate was performed as mentioned before.

b. Results

Radioactivity of C^{14}-sodium acetate treated leucomycin was 25 cpm per mg N and that of C^{14}-starch-leucomycin treated was 15 cpm per mg N. For the estimation of such a low radioactivity, it will be necessary to use a gas-flow windowless radiation-counter.

4. Preparation of C^{14}-leucomycin by biological method.

a. Materials and methods.

A trained female mongrel dog was anesthetized with an intravenous injection of sodium pental, the cystic duct was tied off with braided silk through a medial abdominal incision. The inner part of a lucite Thomes duodenal fistula was placed opposite the duodenal papilla at which the common bile duct opens; The outer part will be inserted in a lateral abdominal incision about one inch to the right of the middle line and one inch from the last rib. Leucomycin tartrate, which assayed 1,010 mcg. of leucomycin activity per. mg., was administrated intravenously in a dose of 150 mg. per kg. body weight. Bile collected was made for the subsequent six hours by cannulation of the common bile duct with the dog lying at rest. Bile was extracted with five equal volume of chloroform and the green chloroform solution was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness under vacuum.

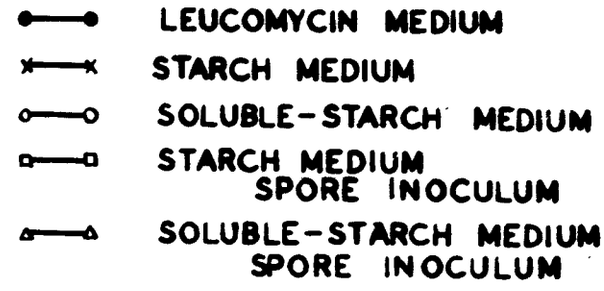
b. Results.

About 80 mg. of light yellow powder, which is assumed de-N-methyl-leucomycin was extracted with chloroform from the dog bile. The natibacterial activity of this powder was 200 mcg per mg. Seventy six mg. of this powder was methylated with CH_3J in methanol and 28 mg solid material was obtained. But this material was assayed the equivalent of only 250 mcg leucomycin per mg. As the amount of the sample was too little, the completion of methylation was not clear, but from its low antibacterial activity, it might not be true leucomycin. This fact will be clarified when C^{14}-methyl-jodid is used in our future experiment.

5. Radioautography

Escherichia coli strain K-12 and Staphylococcus aureus FDA 209-P were employed for this experiment. The organisms were cultured in synthetic media containing all the essential amino acids, purines, pyrimidines, vitamines, glucose as energy source and other minerals. Before the addition of labelled compound, logarhysmic growth had been maintained by shaking at 37 C for several hours. The organisms were harvested by centrifugation, washed once with 0.1 M phosphate buffer pH 7.2 and resuspended in fresh media containing 8-14C-adenine sulfate instead of unlabelled adenine at the conventration of 0.21 micro mol/ml. Then, the incubation was continued in a water bath at 37 C with shaking. One ml of samples were taken at different times and added to the same volume of cold 0.5 N perchloric acid. After the 30 minutes preservation in the cold, the materials were washed and resuspended in 0.1 M phosphate buffer pH 7.2. This suspension was smeared on a slide glass treated with egg albumin solution. Preparation of autoradiogram was carried out with collodion-cadmium bromide method developed by Gomberg et al. The advantage of this procedure is the good resolution obtained.
In the technique of the autoradiography, the time of exposure must be determined empirically. Therefore, the standardization of the procedure is now in process to obtain a constant result.

FIG. I REVERSAL OF PH VALUE



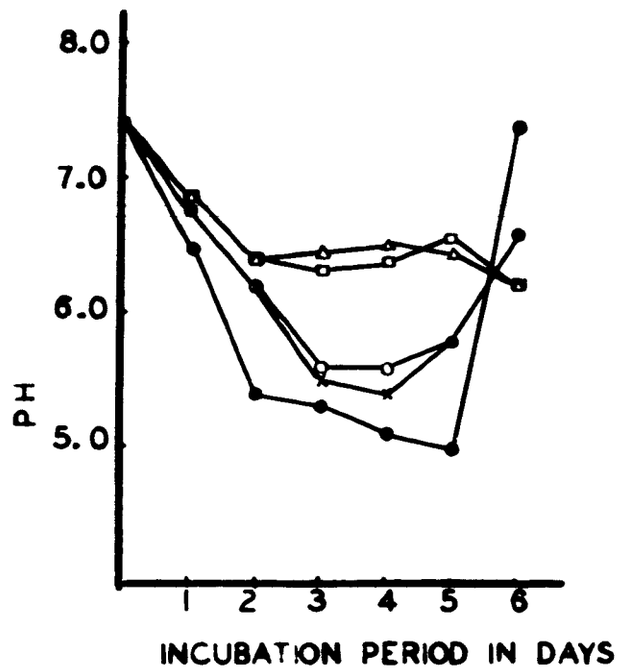


FIG.2 PRODUCTION OF LEUCOMYCIN