ON THE METABOLISM OF CHOLESTEROL AND BETA-SITOSTEROL BY STREPTOMYCES SP.

A Thesis

Presented to

the Faculty of the Department of Biology

The University of Houston

In Partial Fulfillment

of the Requirements of the Degree

Master of Science in Microbiology

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by

Joel Kam Hong Wong

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AN ABSTRACT

The study of the effect of the various nitrogen sources on cholesterol degradation by Streptomyces I_4pH_8, was carried out. Experiments were also performed at different pH values and with different buffer solutions. These results were correlated with those of the nitrogen sources, and it was indicated that a pH of 8.5 gave the optimum growth rate with this organism regardless of the nitrogen source used.

Thiamine and thiamine pyrophosphate were found to enhance the removal of both the 4 carbon and the 26 carbon of the cholesterol molecule to about the same degree.

Streptomyces I_4pH_8 was found to use β-sitosterol as a sole source of carbon. The pathway of degradation of β-sitosterol seems to follow closely that of cholesterol. An intermediate sitostenone was identified.

The presence of β-sitosterol in cholesterol medium did not appear to increase the rate of cholesterol degradation. But, this extra carbon source did increase the growth rate of the organism.

Anumber of steroids were found to be able to serve as the sole carbon source for the growth of Streptomyces I_4pH_8 but two intermediates of cholesterol degradation, cholic acid and Δ^4-cholesten-4ol-3one, did not support growth of the organism.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. G. E. Peterson of the Department of Biology of the University of Houston for his inspiration and encouragement and for his patient guidance towards the completion of this thesis.

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INTRODUCTION

The many roles of cholesterol in mammalian system have been studied and elucidated by many investigators. Of particular interest is its role as steroid hormone (Pincus 1954; Dailey et al 1962), as bile acid (Lindstedt 1962) and as carcinogins (Haven and Bloor 1950; Kennaway 1957). Adlerberg and Sobotka (1958) reviewed the many roles cholesterol may play in human ailments.

In contrast to the large amount of investigation on steroids in mammalian tissues, little is known about microbial metabolism of cholesterol or other steroids. Sohngen (1913) was the first to show that microorganisms are responsible for the decomposition of cholesterol in the soil. Tak (1942) was able to give chemical evidence of this degradation. The classical work of Peterson et al in which microorganisms were demonstrated to be 'experts' in interconversion of steroids has given stimulus to this kind of research. Since then industrial production of valuable adrenocortical hormones has been of great interest.

The same that can be said about cholesterol can be said about β-sitosterol. Although this steroid has been used as a therapeutic compound to lower the serum concentration of cholesterol (Scholenheimer 1929; Peterson 1951) not much is known about its activity. Turfitt (1948) noted that the same microorganisms that are responsible for lowering the cholesterol content in the soil also attack β-sitosterol.

The data in this dissertation are presented in the hope of contri-

buting to the knowledge of microbial degradation of cholesterol, β-sitosterol and other steroid compounds. Also, since photolysis of cholesterol does occur under ordinary experimental conditions, investigations were made to determine whether such an effect is of crucial importance in fermentation experiments. Finally, a general investigation has been made on the various factors (such as nitrogen sources, pH etc) affecting fermentation experiments.

II. LITERATURE REVIEW

Sohngen (1913) first gave evidence that microorganisms played a role in removing cholesterol from the soil. Using growth as the only criterion Sohngen showed that several mycobacteria isolated from the soil with paraffin enrichment media could use cholesterol as a sole source of carbon.

Haag (1927) conducted a series of experiments which supported Sohngen's work. Several soil Mycobacterium species were reported by Haag to cause the disappearance of the cholesterol in the soil.

It was Tak (1942) who gave the first chemical evidence of the degradation of cholesterol in the soil. By precipitation of cholesterol with digitonin he was able to correlate the disappearance of cholesterol with the appearance of cholestenone. Tak used the three organisms, Mycobacterium berolininse, Mycobacterium lacticola and Mycobacterium cholesterlicum in his studies. The latter was showed to pocessed the highest of such activities.

Turfitt (1943) observed that unfavorable soil conditions such as high acidity and waterlogging or lack of aeration caused an accumulation of cholesterol in the soil. Because of the insolubility of cholesterol, he attributed its disappearance to microbial degradation rather than rain erosion. By burying flower pots with cholesterol in the soil and using $HgCl_2$ as a control, he found the most active metabolizer of cholesterol to be gram positive rods which gave raise to coccoid forms. He identified these

organisms as Nocardia sp. He noted that the most active organism was Nocardia erythropolis. Later, Turfitt (1944a and 1944b) reported neither molds nor eubacteria were capable of growth with cholesterol as the sole source of organic carbon. Even Nocardia erythropolis was a slow metabolizer of cholesterol as it took one month for the organism to convert cholesterol to Δ4-cholestene-3one.

In 1946 Turfitt noted that pH change greatly affected cholesterol metabolism. A number of hydroxy-sterols such as β-sitosterol, stigmasterol, testerone and coprosterol were changed to the ketone derivatives by Turfitt's Nocardia erythropolis. However, he obtained no evidence on the method of attack on the cyclopentanoperhydrophenanetherne nucleus.

In another series of experiments performed by turfitt (1948a, 1948b) the halogen substituted steroids were found not to be degradated by Nocardia erythropolis. Using a continuous growth technique and cholestenone as substrate, Turfitt isolated isocaproic acid, Windau's keto acid and 3-keto-etiochol-4enic acid as intermediate compounds of cholesterol degradation. The A ring of the cholesterol was postulated to have been between the C-3 and C-4 position and the side chain of the molecule was removed.

Kramli and Horvath (1947) isolated a species of Azotobacter which metabolised cholesterol as the sole carbon source. Cholestenone, 7-dehydrocholesterol and methylheptanone were isolated as intermediate products. Cholestenone was postulated to be the oxidative degradation product of cholesterol. &-dehydrocholesterol was believed to have resulted from an attack of the B-ring; it was speculated that the methylheptanone was a degradation

product of the side chain. Peterson et al (1962) pointed out the possibility that 7-dehydrocholesterol may be a product from sutoclaving cholesterol.

Schatz (1949) reported that a number of non-motile, gram negative eubacteria attacked the cholesterol molecule. He observed that the isolants attacked more sterols that the N erythropolis isolated by Turfitt.

While looking for circinogenic activities resulting from cholesterol metabolism, Sobel (1949) noted that Mycobacterium smegmatis did not produced cholestenone from cholesterol. He suggested that his organism did not follow the pathway of Turfitt's organism. Talalay, Dobson and Taplay (1952, 1957) used manometric techniques to demonstrate an inducible enzyme in a pseudomonad which attacked a number of hydroxy-steroids and converted them to the corresponding ketone derivatives. A number of other organisms having this same property were also described.

Santer and Ajl (1952) provided with the aid of radioisotopic techniques, followed the catabolism of steroid nuclei. Using 4-C^{14} labelled testosterone and Pseudomonad sp, they demonstrated the presence of radioactivity in dicarboxylic and tricarboxylic acids. Radioactivity was also found in nucleic acids and proteins.

Arnaudi (1949, 1951, 1954) noted that species of Nocardia and Flavobacterium yield cholestene-3-one as a result of cholesterol metabolism.

Stadtman et al (1954) used Schatz's organism, which they reported was actually a gram positive bacillus, to perform radioisotope studies using 4-C^{14}-cholesterol and 26-C^{14}-cholesterol as substrates. They reported the presence of a cell-free cholesterol dehydrogenase which degradated

cholesterol to cholestenone. Also, they found that the decarboxylation at the 4-C position was four times as rapid as that of the 26-C position. Breakdown of the cholestenone was postulated to be done by constitutive enzymes. Testosterone, pregesterone and dehydrolisoandrosterone also supported growth but the organism showed some degree of specificity as ergosterol was not metabolized. Two intestinal organisms Aerobacter aerogenes and Pseudomonas jaejeri were believed by Wainfain (1954) to engage in the metabolism of steroids. In these studies sulfaauxidine and streptomycin which alter the normal flora of the intestine reduced the steroid metabolism of the rats, while both 2, 4-dinitrophenol and nicotinamide stimulated the abilities of both the organisms and the rats to oxidize cholesterol.

Thoma et al (1961) demonstrated a number of microorganisms including Rhodotorula gracilis, a yeast, some Nocardia, Mycobacterium, and Streptomyces species to be capable of converting cholesterol to other steroids.

Lewis (1962) isolated the first Streptomycetes known to use cholesterol as a sole source of carbon. To date, these organisms are the most active metabolizers of cholesterol reported in the literature. The main intermediate product found to be Δ-4-cholestene-3-one, this was metabolised much slower than cholesterol.

Davis (1962) found an extracellular cholesterol dehydrogenase of an inducible nature from the organism isolated by Lewis. This converted cholesterol to cholestenone. Cholic acid also shown by Davis to be one of the intermediates of cholesterol catabolism. Brown (1964) used the organism isolated by Lewis and found an additional intermediate in the

degradation of cholestene-3-one to be Δ-4-cholestene-4ol-3one. Using metabolic inhibitors, Whitmarch (1964) demonstrated that 3-oxo-Δ-4-bisnorcholenic acid and 3-oxo-Δ14-bisnorcholenic acid, Δ4-androstadiene-3, 17-dione andl,4-androstadien-3,17dione to be intermediates of cholesterol metabolism by a Nocardia sp.

Not much is known concerning the metabolic activities of microbes on β-sitosterol. As noted Turfitt (1948) found his organism to be capable of metabolizing β-sitosterol. However, several studies have been made on the animal metabolism of β-sitosterol. β-sitosterol has been often used as therapeutic compound to lower the serum concentration of cholesterol. Scholenheimer (1929) found the feeding of β-sitosterol to rats, mice, guinea pigs, etc. caused liver concentration of cholesterol to be reduced.

Peterson (1951) found that the serum cholesterol of chicks, when elavated by dietary cholesterol, may be lowered again by feeding either β-sitosterol or other plant sterols. Peterson et al (1953) postulated that β-sitosterol inhibited the formation of cholesterol ester in the intestinal mucosa of the experimental animals. However, Glover and Green (1955) postulated that the β-sitosterol at least passed through the mucosa and blocked the intestinal absorption of cholesterol. But, Gould (1955) proved that β-sitosterol at least passed through the mucosa of the intestinal tract to some extent. Beveridge et al (1958) produced evidence that β-sitosterol decreased cholesterol concentration of the serum of human. Finally, Gerson and Shortland (1964) did an extensive experiment

with radioisotopes and found that the β-sitosterol present in an experimental animal can induce a higher rate of overturn of bound cholesterol thus leading to a more rapid metabolism of cholesterol.

III. METERIALS AND METHODS

The two basal media used in these studies were the mineral salts medium (Table 1) and the M-9 synthetic medium (Table 2). The two solutions of the M-9 synthetic medium were autoclaved separately before mixing.

The cholesterol was purchased from the Curtin Scientific Company and the β-sitosterol was donated by Upjohn Company. The other steroids used came from chiefly these two companies or were produced by synthetic means in our laboratory. The cholesterol and the β-sitosterol were purified by the method of Fieser (1953). In this procedure, 100 gm of the steroid were dissolved in 800 ml of boiling acetic acid. The acetic acid was then washed with 500 to 1000 ml of methanol and the crystals were spreaded evenly on a large piece of filter paper and dried overnight. The purified steroid was then recovered by heating in the oven at 90 C until a constant weight was obtained. The purity was tested by thin layer chromatography, spectrophotometry and gas chromatography.

Cholesterol-mineral salts agar plates were prepared according to the method of Peterson, Lewis and Davis (1962). The cholesterol (0.1 to 0,2%) was sterilized dry in a Waring Blender flask at 121.5 C for 15 minutes. The mineral salts solution with 1.5% agar was autoclaved separately and after cooling to about 60 C blended with the cholesterol in the Waring Blender for 2 minutes. The medium was then poured into steril petri or Koole plates. Sitosterol and other steroids of high melting point were prepared in similiar fashion.

Table 1. Composition of mineral-salts solution

COMPOUND	CONCEBTRATION g/l
KNO_3	2.0
K_2HPO_4	0.25
$MgSO_4 \cdot 7\ H_2O$	0.25
NaCl	0.005
$FeSO_4 \cdot 7\ H_2O$	0.0001
Tap water 1000 ml	

Adjust to pH 7

Table 2. Composition of M-9 basal salts solution (Humphrey, 1958)

A. Solution 1

KH_2PO_4	3.00 gm
Na_2HPO_4	9.85 gm
NaCl	0.50 gm
Distilled water	600 ml

B. Solution 2

$MgSO_4 \cdot 7\ H_2O$	0.2 gm
NH_4Cl	1.0 gm
Distilled water	400 ml

Autoclave solutions 1 and 2 separately and mix after cooling

In preparation of fermentation flasks, mineral salts solution without agar was blended with the necessary sterol and dispensed into 250 ml Erlenmeyer flasks. Depending on the experiment, 50 to 100 ml of these steroid-mineral salts solution were added to each flask. Steroids with low temperature of disintegration were sterilized by filtration after being dissolved in an appropriate solvent. The solvent was then evaporated under partial pressure and the steroid under study resuspended in sterile mineral salts solution.

In all experiments requiring submerged cultures, the Erlenmeyer flasks containing 50 to 100 ml of the steroid-mineral salts solution were incubated at 30 C in a New Brunswick Gyratory Shaker which discribed a circle 1 1/2 inches in diameter 150 times per minute. Duplicate flasks were used for each projected collection of sample.

The organisms used in these studies were isolated from the siols of Houston, Texas by the laboratories at the University of Houston. They were classified as Streptomyces sp. by Lewis, Davis and peterson (1962).

Innocula were prepared by either growing the organism on a 0.2 % cholesterol-mineral salts agar. The organism under study was plated from a stock culture on to the surface of the appropriate steroid agar plate. These plates were incubated at 27 C for 5 days. The growth from each plate was used to inoculate three fermentation flasks in the following way. 10 ml of sterile tap water was used to wash the growth off each plate and 2 ml of the resulting spore suspension was used to

inoculate each fermentation flask. In certain cases, inocula were prepared in 50 ml of the steroid mineral salts broth. In these instances, the organisms were incubated on the New Brunswick Gyratory shaker for 4 days. Two ml of resultant growth were then inoculated into each fermentation flask.

Preliminary experimental fermentations were harvested at zero time, at 3, and 6 days. Duplicated flasks were removed at each time and the pH was taken immediately. More detailed experiments were harvested at zero time, 2, 4, and 6 days. Chloroform extractions were performed. The amount of chloroform used depended on the amount of steroid present. For 50-100 mg cholesterol, 25 ml of chloroform was used, and for 10 mg or less, 10 ml chloroform was used. The chloroform layers were separated from the broth, evaporated and stored in the refrigerator for further analysis.

Dry cell weights were obtained by centrifuging 10 ml of the fermentation medium after extraction with chloroform. The supernatant was discarded and the tubes were put into the oven at 100 C overnight.

Cholesterol and sitosterol were determined quantitatively by Sperry and Webb's modification of the Lieberman-Burchard reaction (1950). Samples with 0.1 to 0.5 mg of steroid from the fermentation extracts were dissolved by warming in 1 ml glacial acetic acid. Two ml of chilled reagent consisting of a 19:1 acetic anhydride: 36 N H_2SO_4 were added and the contents of the tubes were allowed to develop color in the dark for 30 minutes before reading on a Beckman Model B spectrophotometer at 620 mu.

Standards of 0.1 - 0.5 mg of the steroid and a reagent blank used for standardization.

The amount of Δ-4-cholestene-3-one produced in fermentation flasks was quantitated by the method of Stadtman et al (1954). To the chloroform extract containing 25-200 ug cholestenone, 2 ml of aldehyde-free ethanol was added and warmed to effect solution. One ml of reagent (0.1% phenylhydrazine in 2 N HCl in ethanol) was added and the tubes placed in a boiling water bath for 5 minutes. After cooling, 15 ml methanol was added into each tube. All tubes were then read on a Beckman Model B spectrophotometer at 390 mu using reagent blanks and standards of 25 to 200 ug cholestenone.

Intermediate products from the fermentation of the steroids, were screened by thin layer chromatography. Thin layer plates were prepared using a Brinckman-DeSaga applicator to apply a layer of Silica Gel H approximately 250 microns in thickness. Plates were then put into an oven at 80 C for 30 minutes. The TLC plates were spotted with chloroform extracts of fermentation and put into a tank for development. A solvent was allowed to advance 10 cm beyond the spots. Four solvent systems were used: 1) chloroform:ethyl acetate, 4:1 (a modification of the solvent of Bennet and Heftmann, 1962); 2) a 9:1 benzene:ethanol solution; 3) chloroform: methanol:water, 1:1:1 (opcit, 1962); and 4) chloroform: 1N NH_4OH, 1:1.

The thin layer chromatography plates were spayed, after drying for the detection of ketone-containing intermediates with a phenylhydrazine spray reagent. A 5% phosphomolybdic acid in absolute ethanol was used for

the detection of hydroxyl derivatives. The phenylhydrazine spray was made by dissolving 3 gm of 2,4-dinitrophenylhydrazine in 15 ml of concentrated H_2SO_4. Then 20 ml distilled water was carfully added. The resulting solution was added to 70 ml of 95% aldehyde-free ethanol (prepared by refluxing commercial 95% ethanol with zinc dust and NaOH for 4 hr. and distilling to half volume). In some studies, a phenol red-borate buffer, as described by Pan (1962) was used for the detection of acidic intermediates. The suspected intermediates were always checked chromatographically against known standards.

Intermediate products were looked for using a Beckman DK2 spectrophotometer. Chloroform extracts of broth were evaporated to dryness and the residue was dissolved in ethanol and in chloroform. The spectrophotographic absorption was studied in the UV region (320-220 mu). In addition, the water soluble portions of the fermentation broths were scanned in both the UV and the infra-red region. Standards of known compounds were used for comparision.

Gas chromatography was done witn a Barber Colman 5320 Gas chromatographer. A six feet 1% QF1 80-100 mesh Gas Chrome Q column was used. Known steroid samples were used as standards.

Cell-free β-sitosterol dehydrogenase activity of Streptomyces I_4pH_8 was investigated in the following manner. Cells were grown in fermentation flasks containing 50 ml of a sitosterol-mineral salts broth for 4 days and centrifuged. The supernatant was then inoculated into fermentation flasks containing 100 ml sitosterol-mineral salts solution.

The breakdown products of the steroid were analysed with the techniques mentioned previously.

The effects of certain nitrogen sources on cholesterol metabolism were also studied. In three experiments, KNO_3, NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$ were used separately as sole nitrogen sources in the mineral salts solution. The pH change, the dry cell weights and the utilization of cholesterol were followed in these experiments. A set of parallel experiments was done with M-9 synthetic medium, this time using NH_4Cl as the sole carbon source. The results of these two sets of experiments were compared.

The effect of pH in the 2 different broths on the metabolism of cholesterol by Streptomyces I_4pH_8 were studied. By using a phosphate buffer the initial pH of the mineral-salts solution were adjusted to different points from 5 to 10. The adjustments of the pH values were done by adding HCl or NH_4OH aseptically to a sterilized and cooled mineral salts solution. A daily check on the pH was done, the dry cell weights were used as a measure of growth, and these data were related to the substrate utilization. For comparison purposes, the pH changes of the M-9 synthetic medium were also studied from pH 7 through 9. The dry cell weights and substrate utilizations were checked every two days. The pH change with this mineral solution was compared with that of the mineral salts solution.

Effects of thiamine and thiamine pyrophosphate of decarboxylation activities of Streptomyces I_4pH_8 were investigated by adding a sterile

0.1 molar solution of each into fermentation flasks containing the blended cholesterol mineral salts broth. The utilization of the substrate as well as the increases in dry cell weights were checked every day for six days. Detailed studies of the effect of thiamine and thiamine pyrophosphate on cholesterol decarboxylation were made using the Warburg manometric apparatus. The Inocula for the Warburg studies were grown in a 2% glucose solution for 2 days, washed two times in a sterile buffer and grown for another two days in a 2% cholesterol solution. Preliminary experiments were carried out with unlabelled cholesterol and varying amounts of thiamine and thiamine pyrophosphate. From the results of these experiments, experiments with radioactive cholesterol were planned. The dose of labelled substance was estimated from previous experiments by Davis (1962). A dose of 6 lambda of 26 C^{14} (sp. activity 810 cpm per lambda) and 18 lambda 4 C^{14} (sp. activity 3240 cpm per lambda) were used in each flask. A piece of filter paper 1 in^2 folded four times was put into the center well of each Warburg flasks along with 0.1 ml of a 20% KOH solution to capture the CO_2 given off during growth and throughout the Warburg experiments. All procedures were done according to Manometric Techniques (Burris, Umbreit and Stauffer).

The contents of each of the manometer flasks in the preliminary and the radioactive experiments are shown in Table 3.

Table 3. Contents of warburg-flasks in thiamine and thiamine pyrophosphate experiments

Flask	Phosphate buffer	Cell	20% KOH	Cholesterol	Thiamine	TPP
Thermo-bar	3 ml	____	0.1 ml	_____	_____	_____
Thermo-bar	3 ml	____	0.1 ml	_____	_____	_____
Endogen-ous	2 ml	1 ml	0.1 ml	_____	_____	_____
Endogen-ous	2 ml	1 ml	0.1 ml	_____	_____	_____
Choles-terol	_____	1 ml	0.1 ml	2 ml	_____	_____
Choles-terol	_____	1 ml	0.1 ml	2 ml	_____	_____
Chol + Thiamine	_____	1 ml	0.1 ml	2 ml	0.1-o.3M mole	_____
Chol + Thiamine	_____	1 ml	0.1 ml	2 ml	0.1-0.3M mole	_____
Chol + TPP	_____	1 ml	0.1 ml	2 ml	_____	0.1-0.3 M mole
Chol + TPP	_____	1 ml	0.1 ml	2 ml	_____	0.1-0.3 M mole

Sample preparations were made according to the following method. Two flasks each of the labelled cholesterol and labelled cholesterol plus thiamine or thiamine pyrophosphate were taken off after 90 minutes and 180 minutes of experimentation. The pieces of filter paper with CO_2 were put into acid-cleaned test tubes containing 20 ml of 20% KOH solution. Three ml of 0.2 M BaCl and 2.9 ml 10% K_2CO_3 were then added to each tube. After heating to boiling for 10 minutes they were cooled and the precipitate was washed with distilled water and acetone onto a center metallic disc filter on which a layer of celite (20 mg) was deposited. The samples were counted with a GM counter (Model 181A) for five minutes each.

The relationships between respiration and duration of starvation were also studied in the Warburg apparatus. Cells were starved for 12, 24, 36, and 48 hours, respectively, and their endogenous respiration was determined. Each manometric flask contained the same contents as the endogenous flasks described for the previous experiments.

Another experiment was done to investigate if thiamine and thiamine pyrophosphate could be used as a sole carbon source by Streptomyces I4pH8 No. 2. In these flasks 2 ml of 0.1% of thiamine or thiamine pyrophosphate were added instead of the usual cholesterol.

Hais and Masters (1964) noted considerable photolysis of cholesterol during biological experiments. Therefore we studied the photolysis of cholesterol in the following manner. Purification of commerical cholesterol was done with Fieser's method as mentioned. Cholesterol of higher purity was prepared by digitonide precipitation. Each of these samples

were put into: 1) a brown bottle on the laboratory bench; 2) a transparent bottle on the laboratory bench; 3) a brown bottle in the refrigerator at 4 C; 4) a transparent bottle at 4 C; 5) a chloroform solution on the shaker; and, 6) a cholesterol-mineral-salts blended solution on the shaker. These samples were checked after 2 days, 4 days, 6 days, 2 weeks, and 2 months for product of photolysis. Three methods, thin layer chromatography, gas chromatography and UV spectrophotometry were employed for checking the purity of the cholesterol sample after periods mentioned.

The effects of β-sitosterol on the degradation of cholesterol by Streptomyces I_4pH_8 No. 2 were investigated using a 0.1% sitosterol in a 0.9% cholesterol mineral-salts solution. A 10% cholesterol-mineral salts solution was used as the control. The dry cell weights and the utilization of the steroids were follwed. More detailed experiments of this nature were also performed in the Warburg apparatus with radioactive C 4 and C 26 labelled cholesterol.

The contents of each of the Warburg flasks in these experiments are as in Table 4. The experimental techniques and methods of recovering and counting the radioactive CO_2 were as described previously.

Table 4. Contents of warburg-flasks in experiments on the effect of beta-sitosterol on cholesterol metabolism

Flask	Phosphate Buffer	Cell	20% KOH	Cholesterol	Sitosterol
Thermobar	3 ml	____	0.1 ml	_____	_____
Thermobar	3 ml	____	0.1 ml	_____	_____
Endogenous	2 ml	1 ml	0.1 ml	_____	_____
Endogenous	2 ml	1 ml	0.1 ml	_____	_____
Cholesterol	_____	1 ml	0.1 ml	1.8 ml	0.2 ml
Cholesterol	_____	1 ml	0.1 ml	1.8 ml	0.2 ml
Cholesterol plus Sitosterol	_____	1 ml	0.1 ml	1.8 ml	0.2 ml
Cholesterol plus Sitosterol	_____	1 ml	0.1 ml	1.8 ml	0.2 ml

IV. RESULTS

The different ammonium salts had generally the same effect on the catabolism of cholesterol in the cholesterol-mineral salts medium. In each case there was approximately a 60% utilization of cholesterol, and the pH dropped from 7 to 3.5 - 5.0 during the six day experimental period. However, when KNO_3 was the sole nitrogen source, over 80% of the cholesterol was utilized and the pH, instead of dropping, rosed in 4 days from 7 to around 8.5, where it remained for the rest of the experimental period. These results are in agreement with those of Lewis (1961). The production of cholestenone was checked in each of these experiments, and was found to correlate with the utilization of cholesterol.

It seemed likely that the pH changes in these experiments, rather than the kind of nitrogen, per se, was affecting the amount of the cholesterol used. Thus an experiment was designed to investigate into the effect of pH on cholesterol utilization. KNO_3 was used as the sole nitrogen source and a phosphate buffer was employed to adjust the initial pH through a range of 5 to 10. The adjustment of the pH was done after sterilization of the cholesterol-mineral salts solution at pH7. This was done to avoid the accumulation of anions (thus lowering of pH) from the breaking down of various inorganic salts during autoclaving. The pH adjustments were accomplished by adding the correct amount of sterile 2 N HCl or 2 N NH_4OH aseptically. The pH values of the flasks were checked initially and after the third and the sixth days of fermentation. Regardless of the

Figure 1. THE EFFECTS OF NITROGEN SALTS ON CHOLESTEROL UTILIZATION BY STREPTOMYCES I_4pH_8

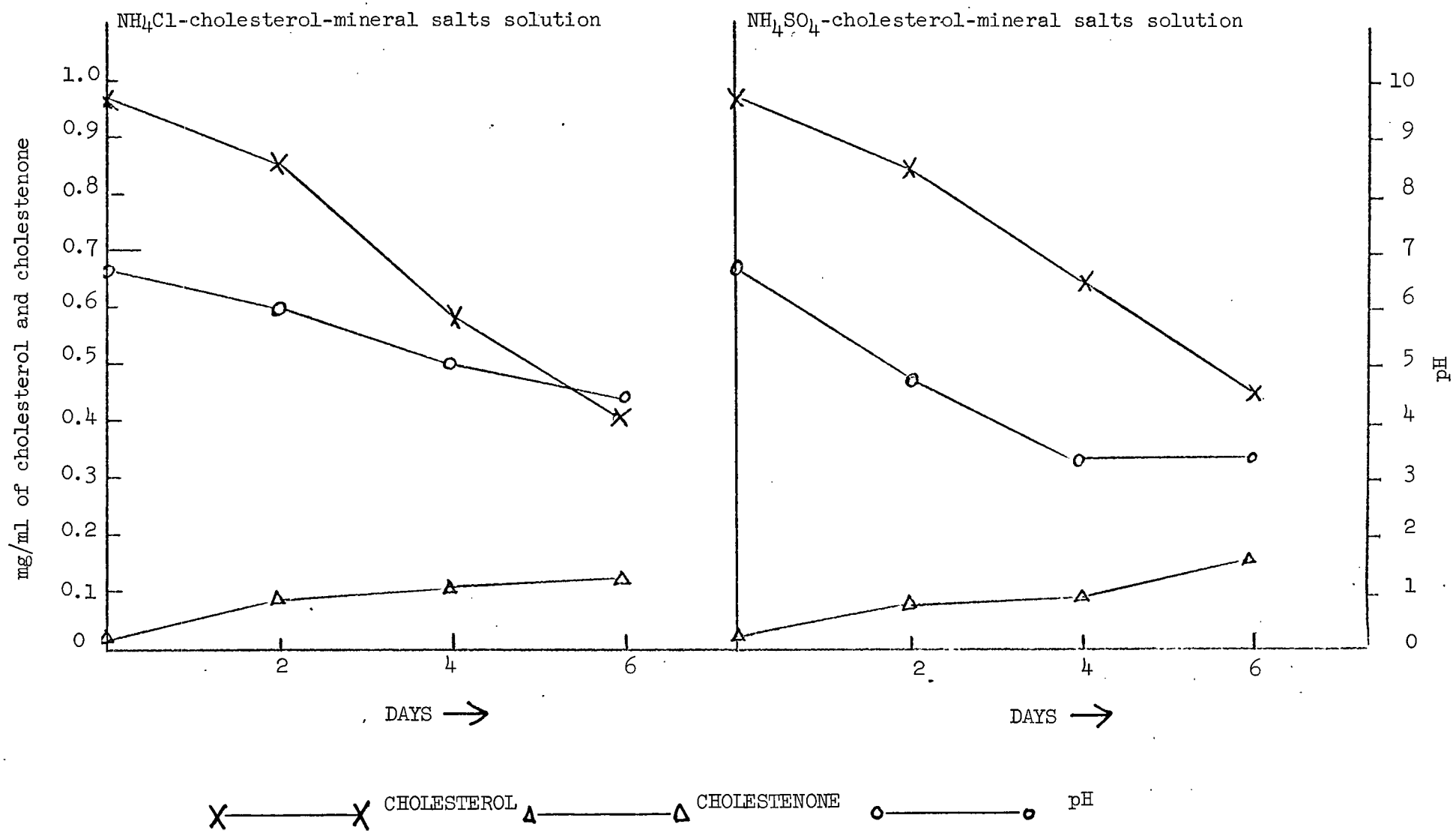
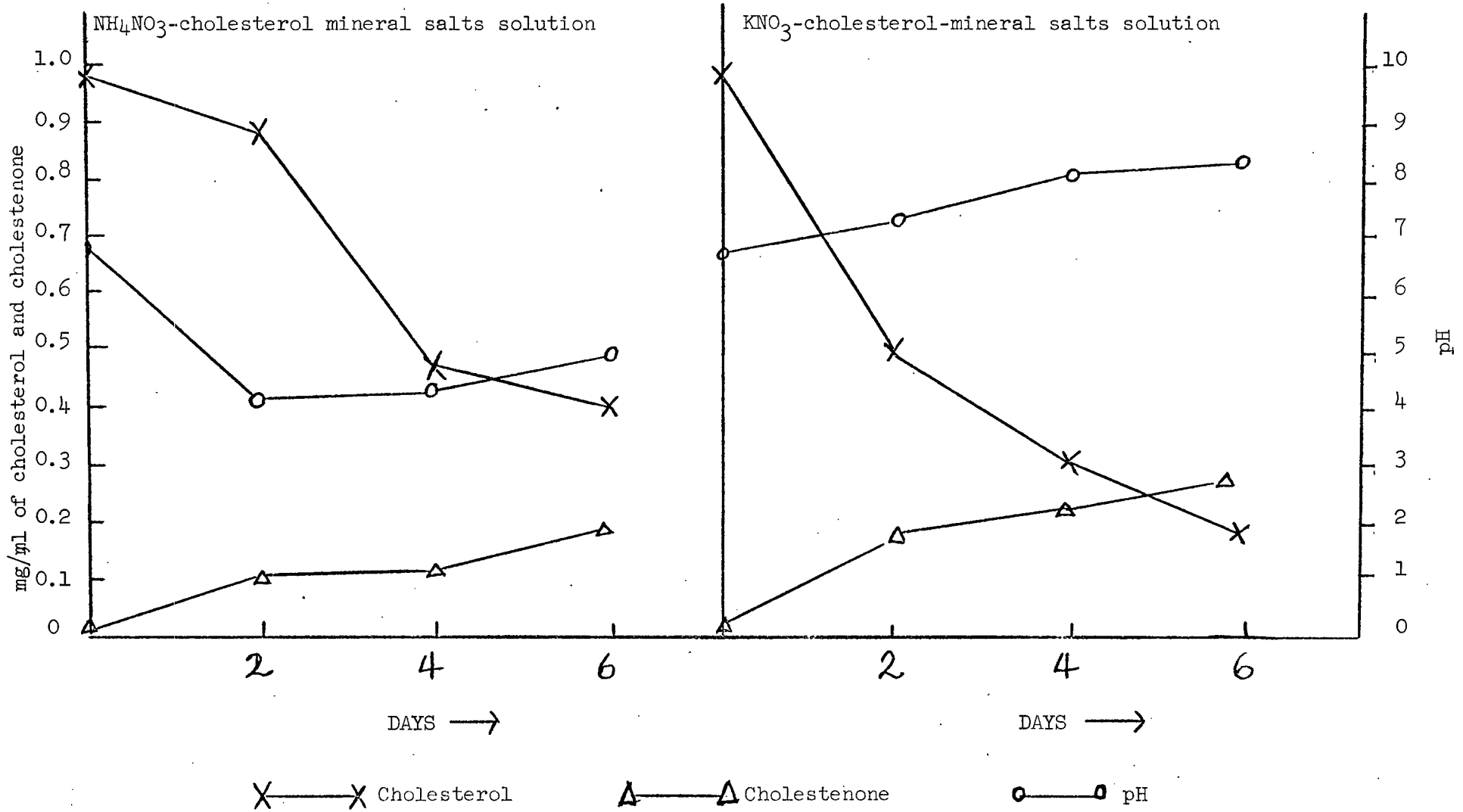


Figure 2. THE EFFECTS OF NITROGEN SALTS ON CHOLESTEROL UTILIZATION BY STREPTOMYCES I_4pH_8



initial pH, the pH values in each flask changed to around 8.5 after 3 days and stayed there until the end of the experiment. These changes were probably not due to some physical effects because the uninoculated control showed a stable pH7 throughout the entire experiment (Fig. 3). The amount of cholesterol utilized as well as the dry cell weights were also checked at the end of the six-day period. It was found that the fermentation flasks with initial pH values of 8 and 9 showed the greatest amount of cholesterol utilized (Fig. 4). The greatest dry cell weights were also observed in the flasks adjusted to pH8.

In order to study further the effect of pH on cholesterol metabolism by this Streptomyces I_4pH_8 , a different mineral salts medium was used. The M-9 synthetic medium which contains two phosphate salts and is therefore much better buffered medium than the cholesterol-mineral salts medium for these experiments. During this study, the nitrogen source used in both the mineral salts and the M-9 synthetic media was KNO_3 and the pH values were adjusted through a range of 7 - 9. After three days the culture in the M-9 synthetic medium showed a much more rapid utilization of cholesterol as well as a better cell growth. Also, the cells grown at pH8 in both media showed higher substrate utilization and dry cell weight signifying that the cells grew better at pH8 than pH7 or (Fig. 5).

Figure 3. pH CHANGES DURING CHOLESTEROL FERMENTATION BY STREPTOMYCES I_4pH_8 IN MINERAL SALTS SOLUTION

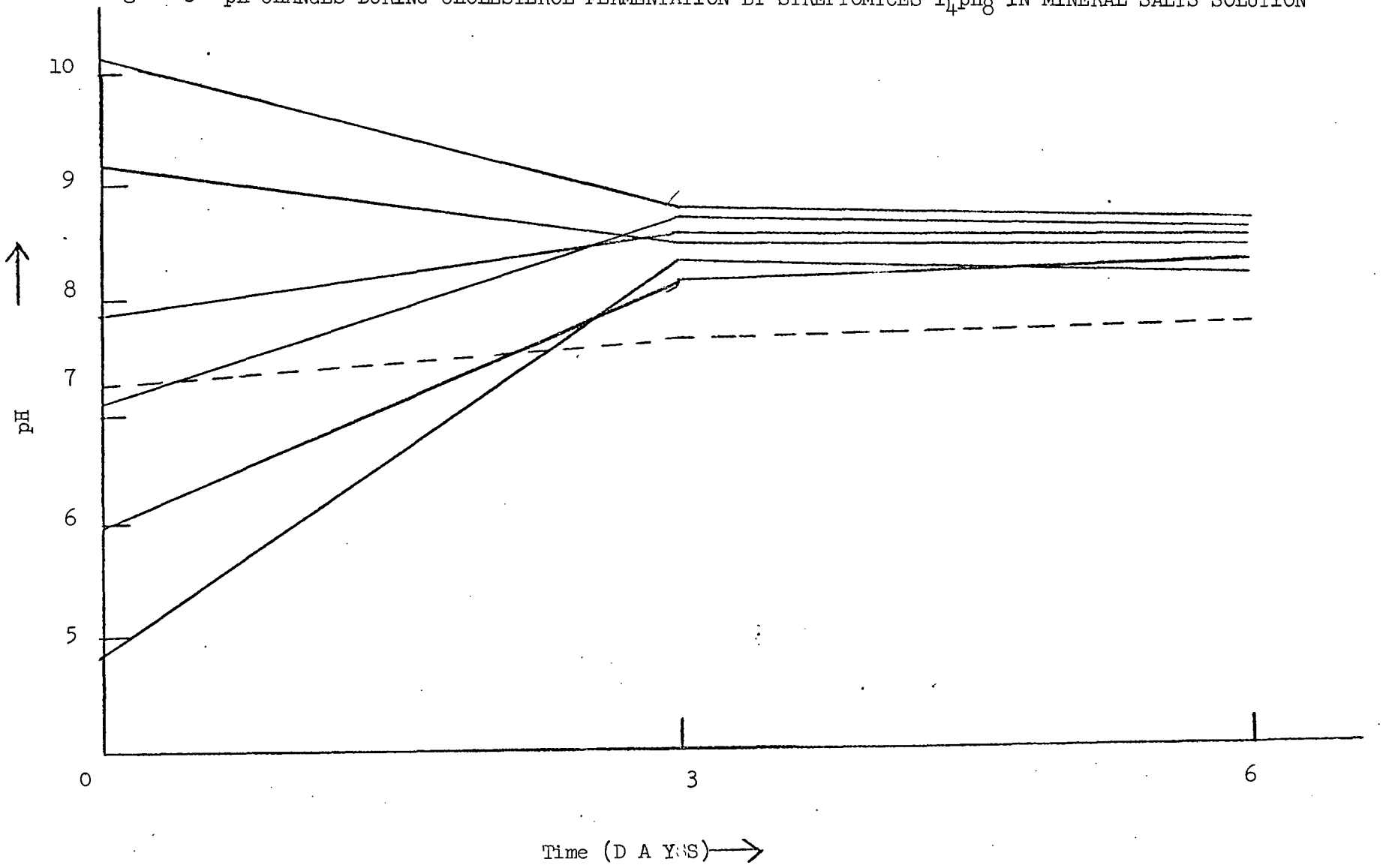


Figure 4. CHOLESTEROL UTILIZATION AT DIFFERENT pH BY STREPTOMYCES I_4pH_8 IN MINERAL SALTS SOLUTION

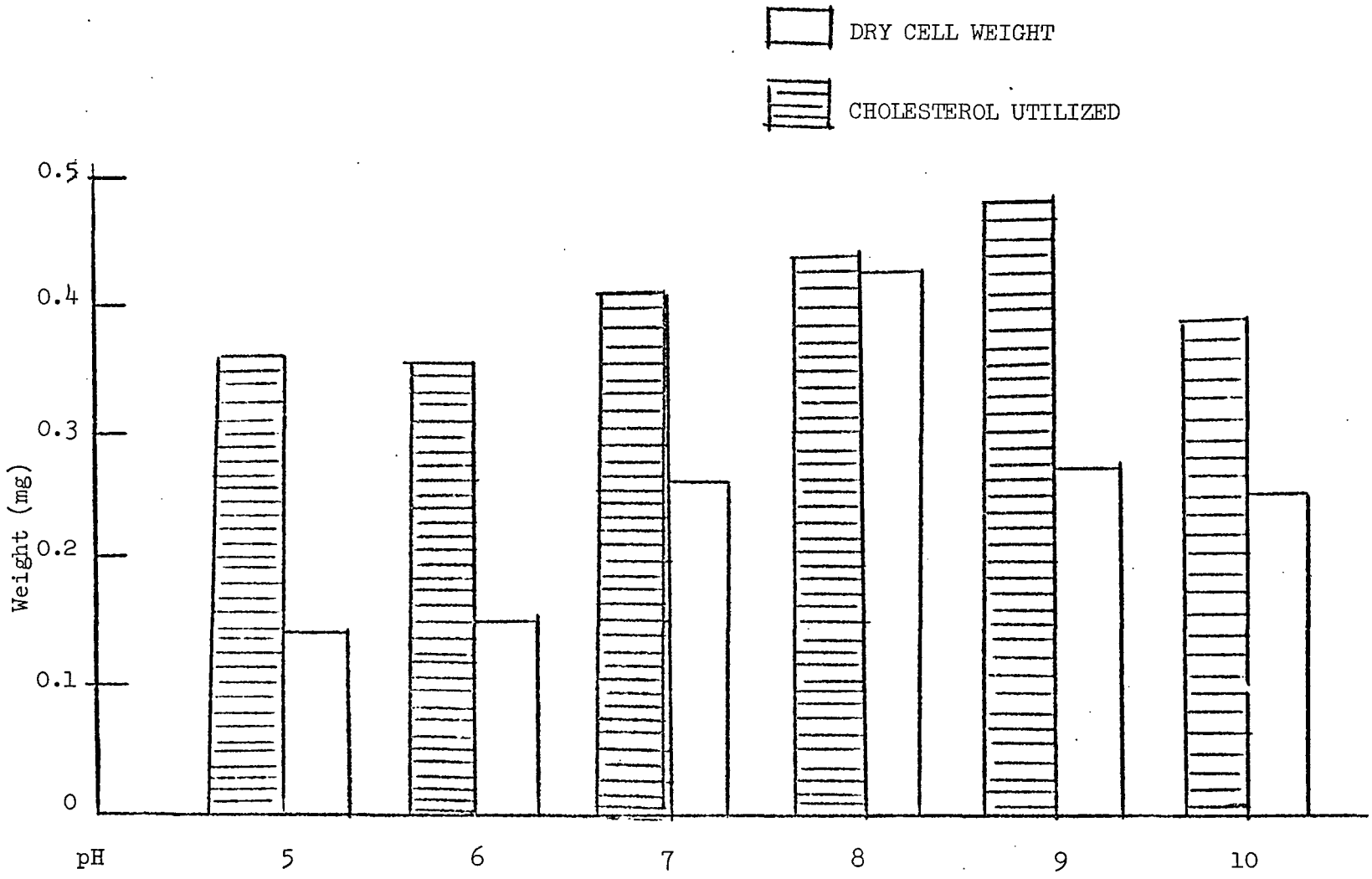
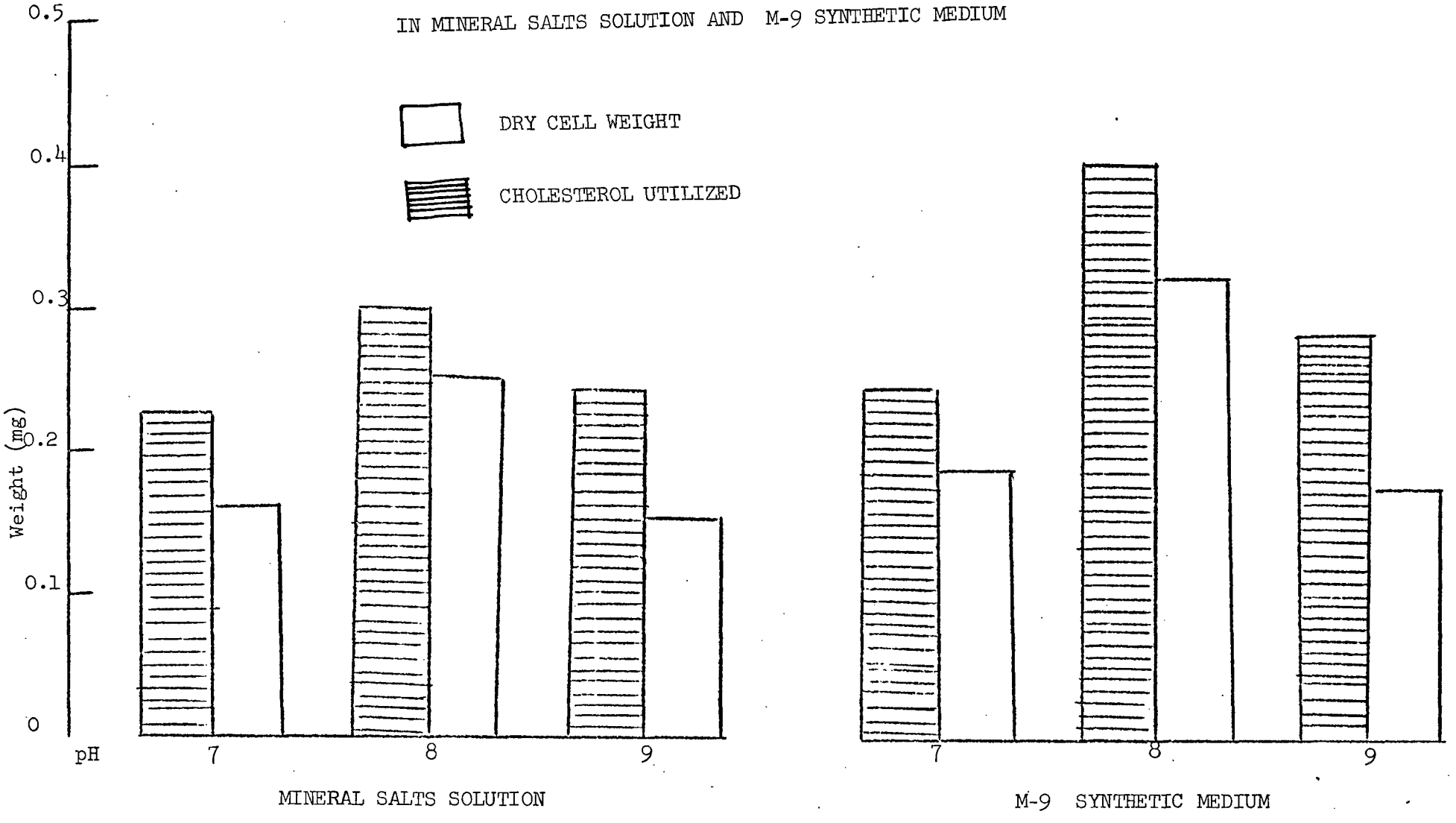


Figure 5. COMPARISION OF CHOLESTEROL UTILIZATION BY STREPTOMYCES I_4pH_8 AFTER GROWTH FOR 3 DAYS IN MINERAL SALTS SOLUTION AND M-9 SYNTHETIC MEDIUM



In the studies involving different nitrogen sources, pH adjustments, routine checks for metabolic intermediates of cholesterol catabolism were made. Both the water phase and the chloroform extracts of fermentations were analysed by thin layer chromatography and spectrophotometric techniques. On the thin layer plates unidentified spots appeared along with the identified spots caused by the intermediates cholestenone nad cholesten-4ol-3one. However, the ultraviolet light and visible light scans on the spectrophotometer showed only the two identified intermediates (Fig 6 & 7).

The effect of thiamine and thiamine pyrophosphate on cholesterol metabolism by submerged cultures of Streptomyces I_4pH_8 showed that both thiamine and thiamine pyrophosphate caused a more rapid substrate ultilization as well as a higher dry cell weight. Further, the organism Streptomyces I_4pH_8 had been demonstrated in another series of experiments to be able to use thiamine and thiamine pyrophosphate as sole carbon sources as evidenced by growth of the organism (Fig 9).

These experiments with thiamine and thiamine pyrophosphate were studied in more detail using the Warburg Manometer and labelled 4-C^{14} and 26-C^{14} cholesterol in the presence and absence of thiamine and thiamine pyrophosphate. There were some technical difficulties encountered. Cells after repeated transfers in cholesterol-mineral salts solutions were found to be"weakened" in their abilities to utilize cholesterol but fortunately periodic transfers to glucose-mineral salts medium restores their ability to use cholesterol (Table 5). It was found that about

Figure 6. ULTRAVIOLET ABSORPTION SPECTRUM OF CHOLESTEROL FERMENTATION AFTER A 6-DAYS GROWTH OF STREPTOMYCES I_4pH_8

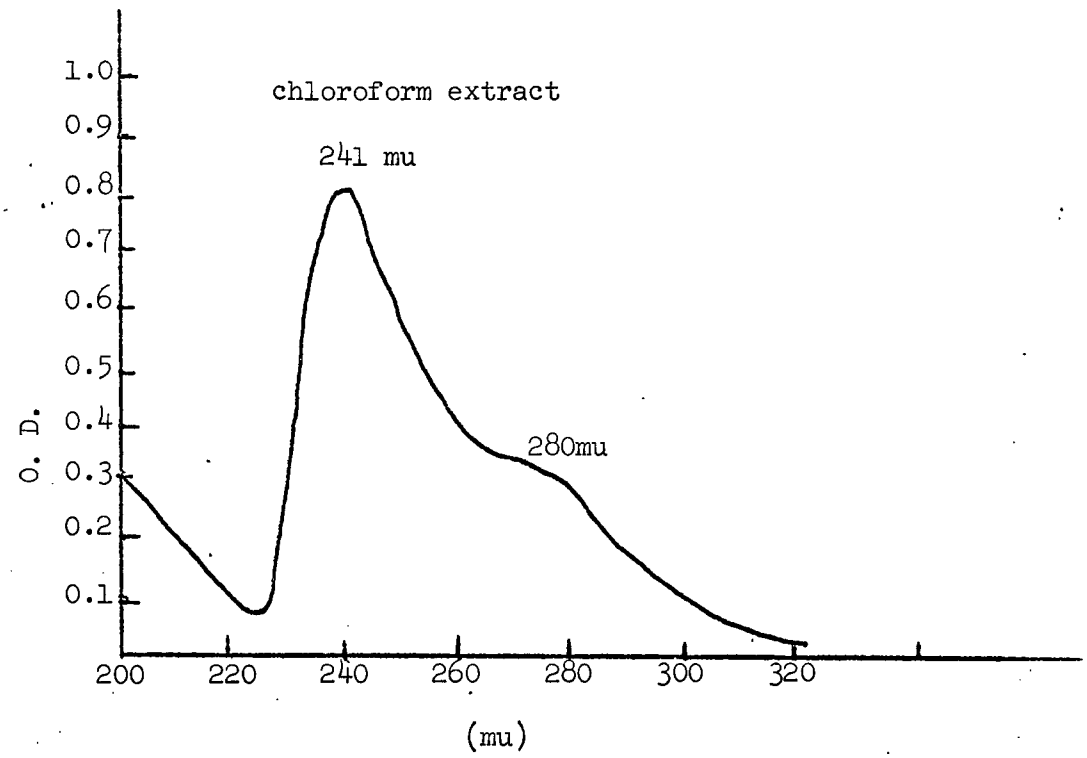


Figure 7. THIN LAYER CHROMATOGRAPHY OF A 3-DAYS FERMENTATION CHLOROFORM EXTRACT OF CHOLESTEROL BY STREPTOMYCES I_4pH_8

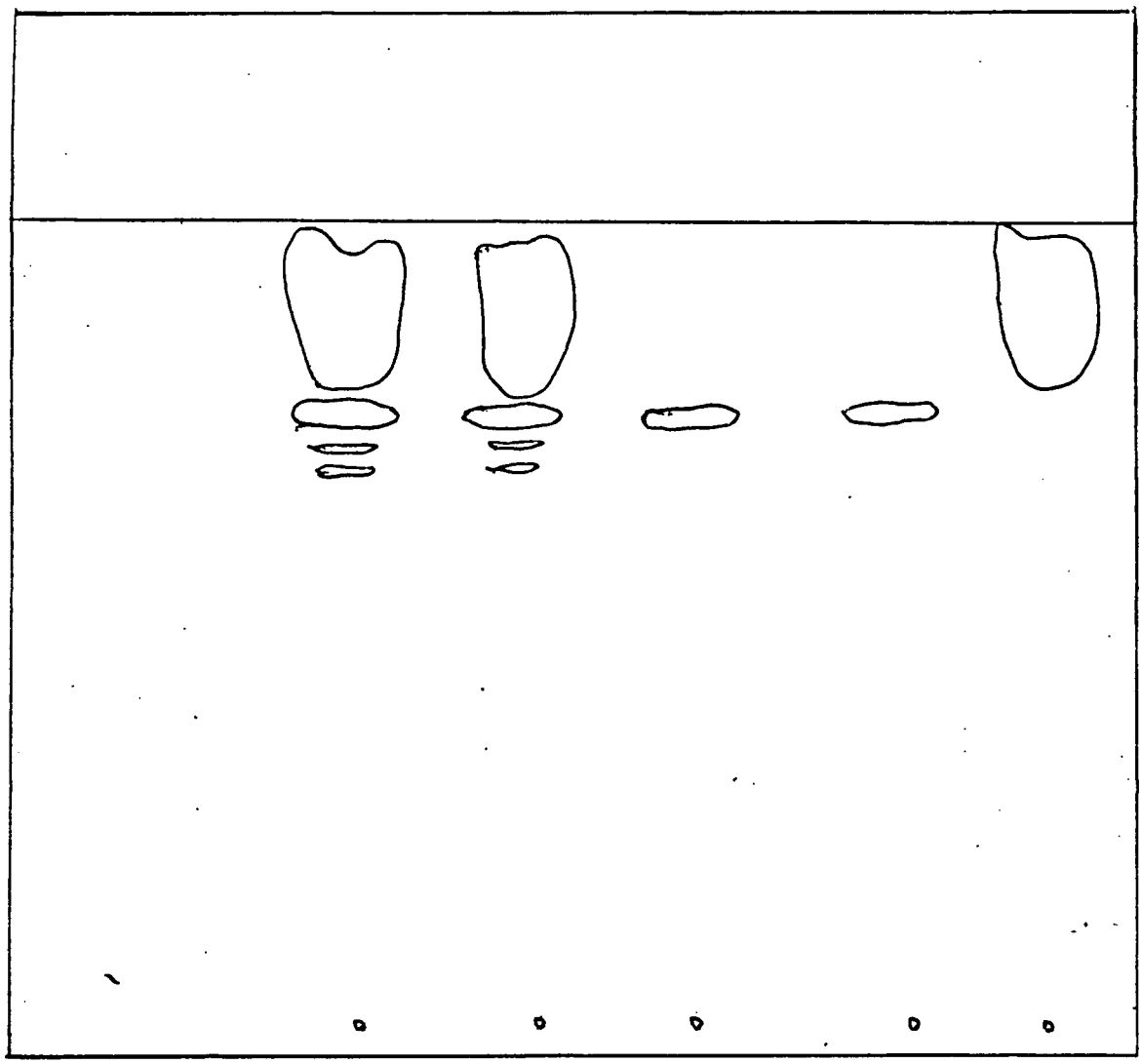


Table 5. A comparasion between cell growth of Streptomyces I_4pH_8 in cholesterol for 6 days and in glucose for 2 days then transferred to cholesterol for an additional 2 days

Cells grown for 6 days in cholesterol. Cell weight/ml

Experiment No.	1	2	3	4	5	6	Average
	0.0071	0.0065	0.0073	0.0059	0.0072	0.0049	0.0065

Cells grown for 2 days in glucose and 2 days in cholesterol. Cell weight/ml

Experiment No.	1	2	3	4	5	6	Average
	0.0142	0.0129	0.0149	0.0148	0.0153	0.0153	0.0145

14 mg of dry cells per ml must be present in order to obtain good results with the Warburg. In order to produce such a population of cells, the inoculum was prepared by first growing in glucose-mineral salts solution for 2 days and washing and resuspending the growth in cholesterol-mineral salts solution for additional 2 days. This method of preparing the inoculum for Warburg studies provided a constant high population of cells (Table 5). Before each experiment the cells were harvested, washed twice in sterile phosphate buffer, resuspended and starved for 36 hr on the shaker. This period of starvation proved to be critical as inadequate starvation gave a high endogenous respiration and too long a starvation period lead to the autolusis of the cells (Fig 8).

Thiamine and thiamine pyrophosphate can be metabolized as sole carbon sources. This was shown by substituting these materials for cholesterol in the Warburg Manometric flasks (Fig 9). Although the oxygen uptake was not as high with these substrates as with cholesterol, they can never-the-less be broken down by this particular organism.

By using radioactively labelled 4-C^{14} and 26-C^{14} cholesterol, it was shown that both thiamine and thaimine pyrophosphate had increased the oxygen uptake and the decarboxylation actively of Streptomyces I_4pH_8 (Fig 10-13). The decarboxylation of the 26-carbon was approximately twice as fast as that of the 4-carbon in both cases. There was no difference observed between the effect of thiamine and that of thiamine pyrophosphate.

Figure 8. EFFECT OF STARVING ON ENDOGENOUS RESPIRATION OF STREPTOMYCES I_4pH_8

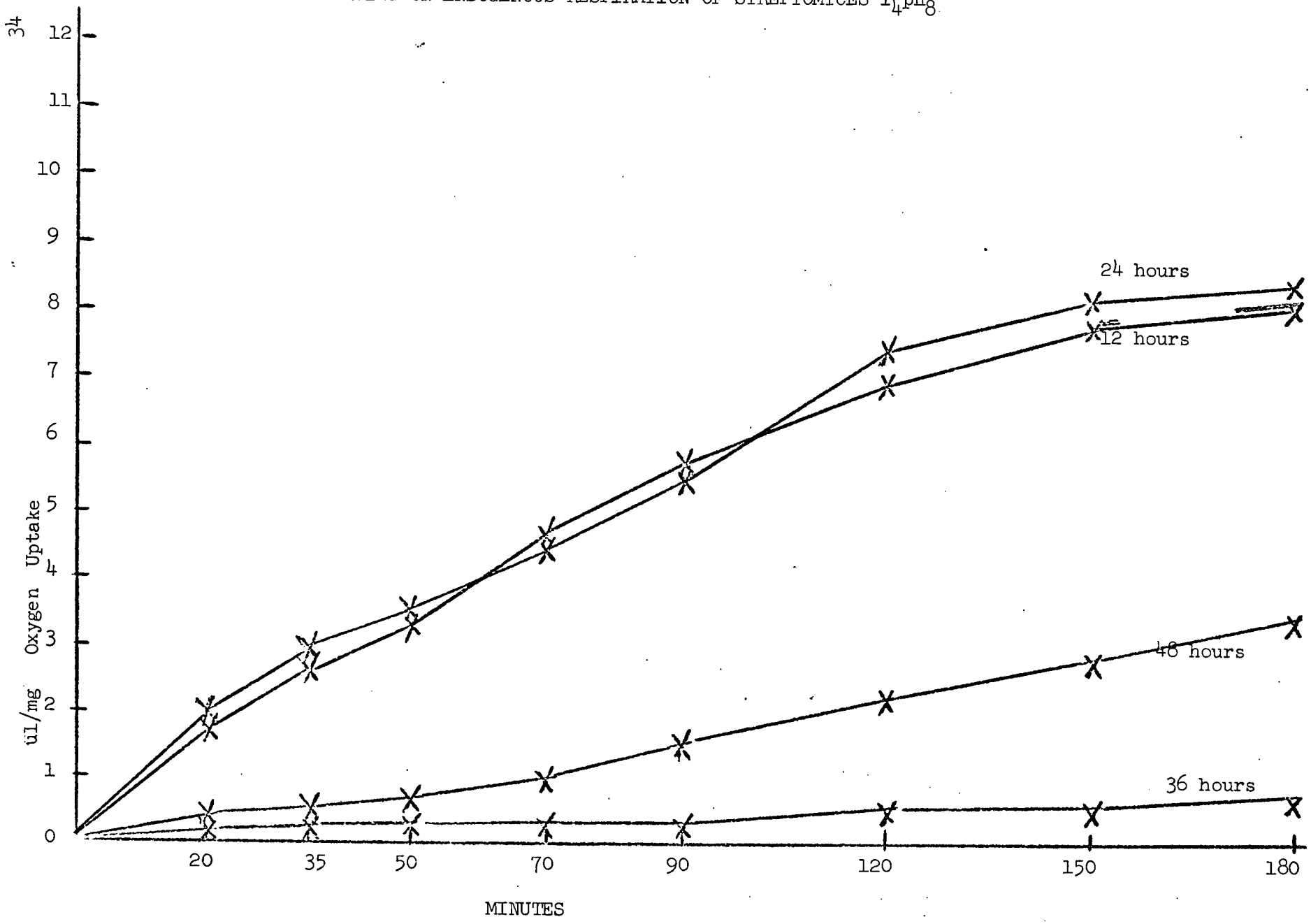


Figure 9. OXYGEN UPTAKE OF STREPTOMYCES I_4 pH_8 USING THIAMINE AND THIAMINE PYROPHOSPHATE AS SOLE CARBON SOURCES

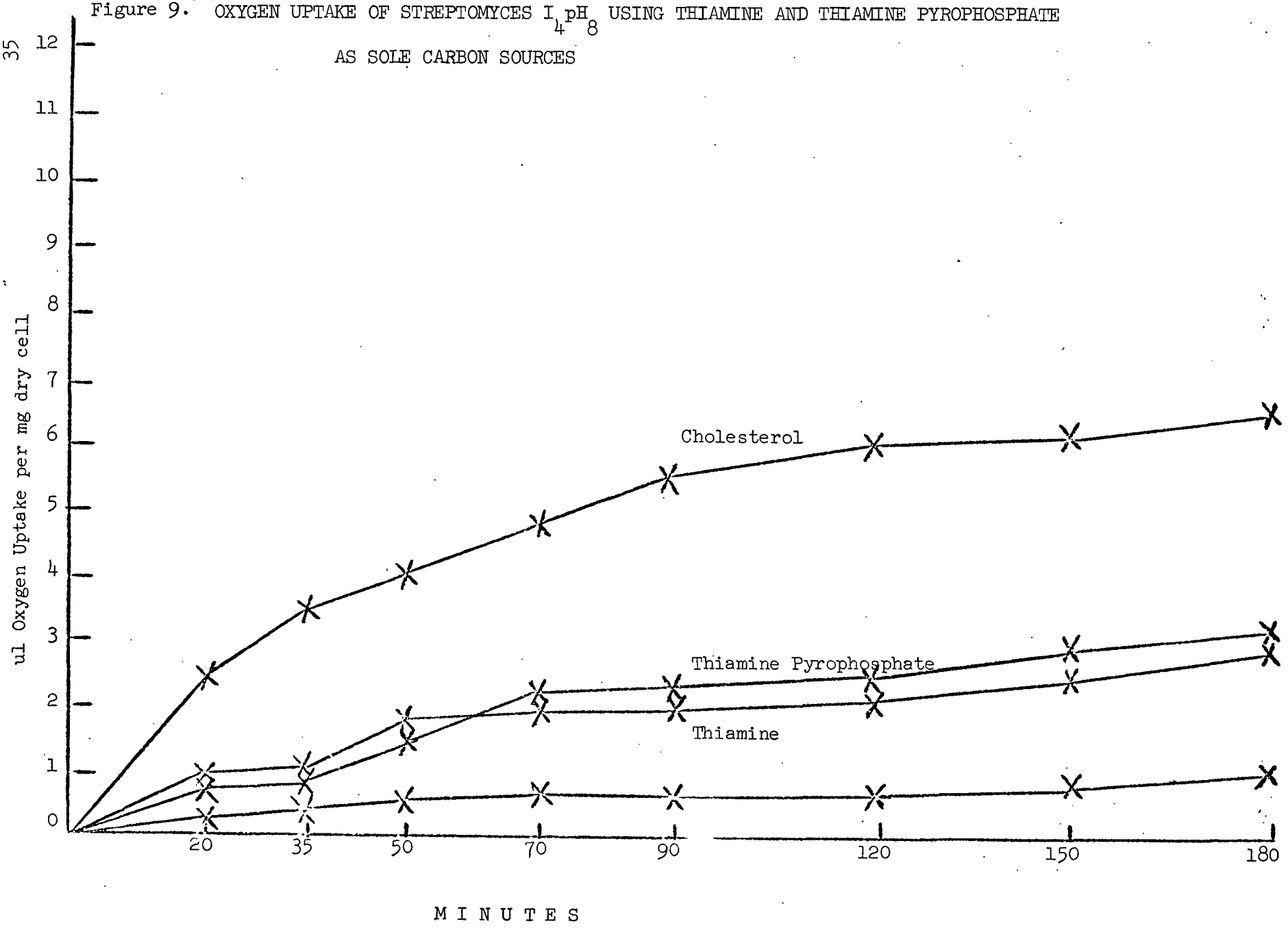


Figure 10. EFFECT OF THIAMINE ON THE DECARBOXYLATION ACTIVITY OF STREPTOMYCES I_4pH_8 ON 26 C LABELLED CHOLESTEROL

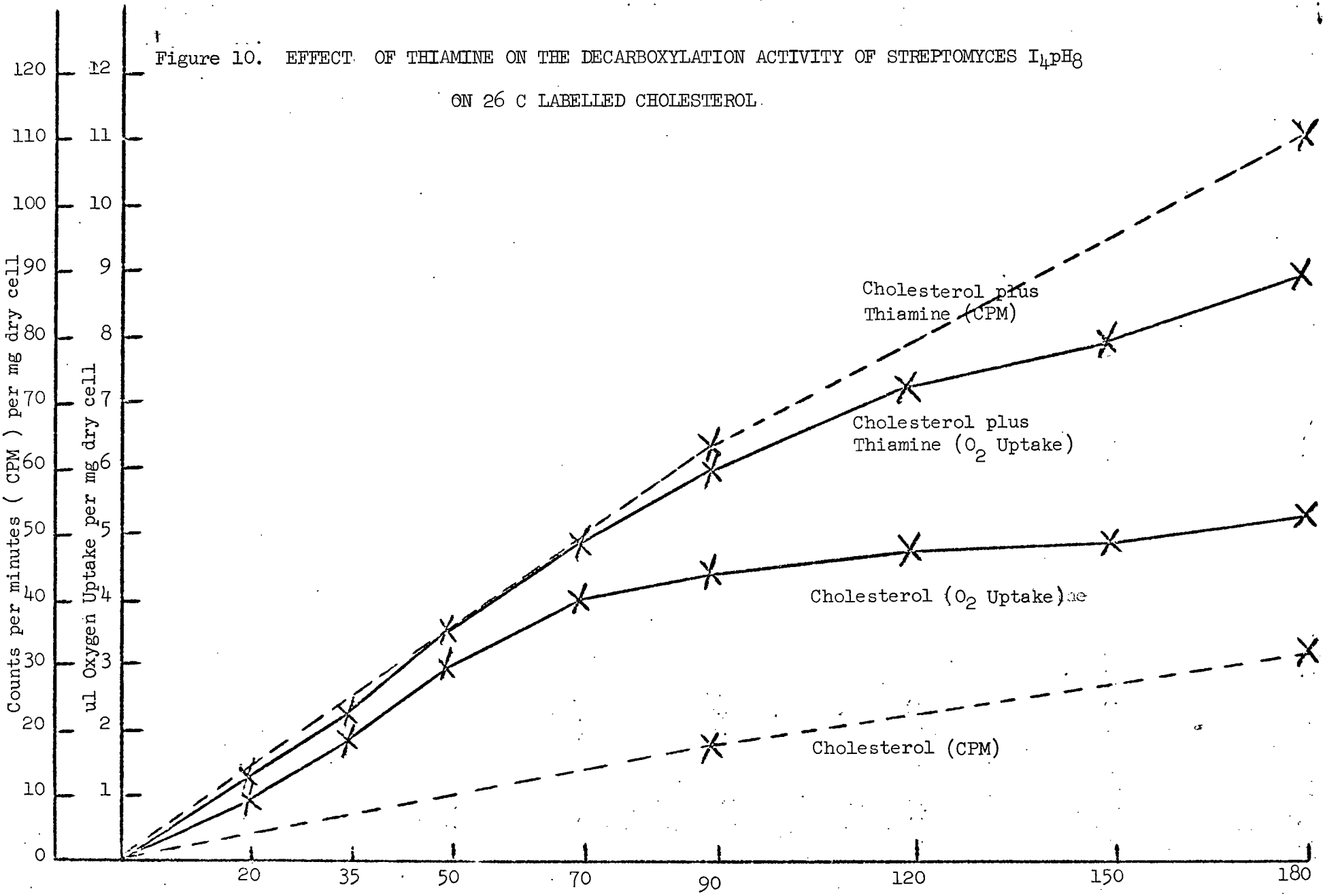


Figure 11. EFFECT OF THIAMINE PYROPHOSPHATE (TPP) ON THE DECARBOXYLATION ACTIVITY OF STREPTOMYCES I_4pH_8 ON 26 C LABELLED CHOLESTEROL

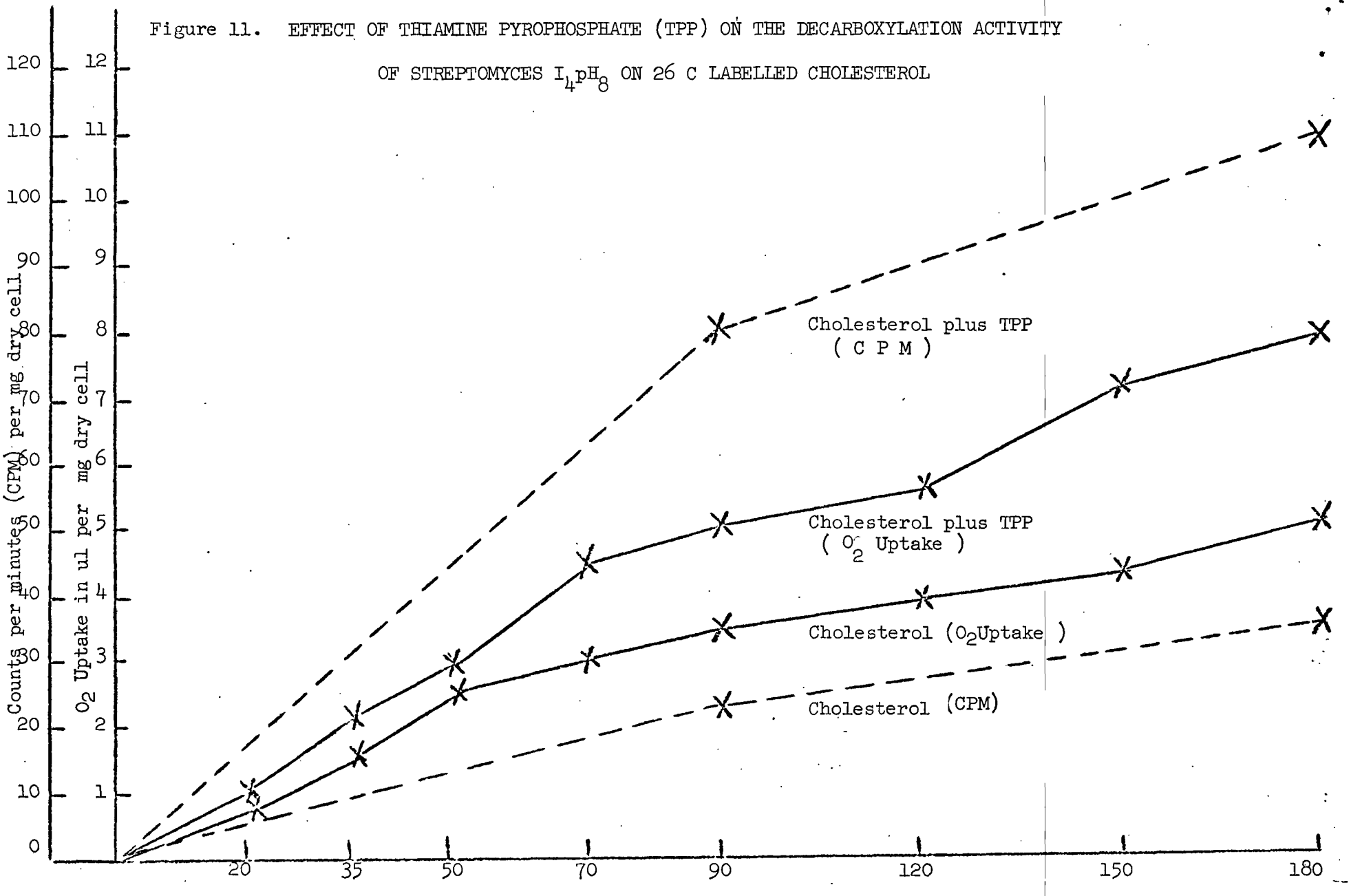


Figure 12. EFFECT OF THIAMINE ON THE DECARBOXYLATION OF STREPTOMYCES I_4pH_8 ON 4 C LABELLED CHOLESTEROL

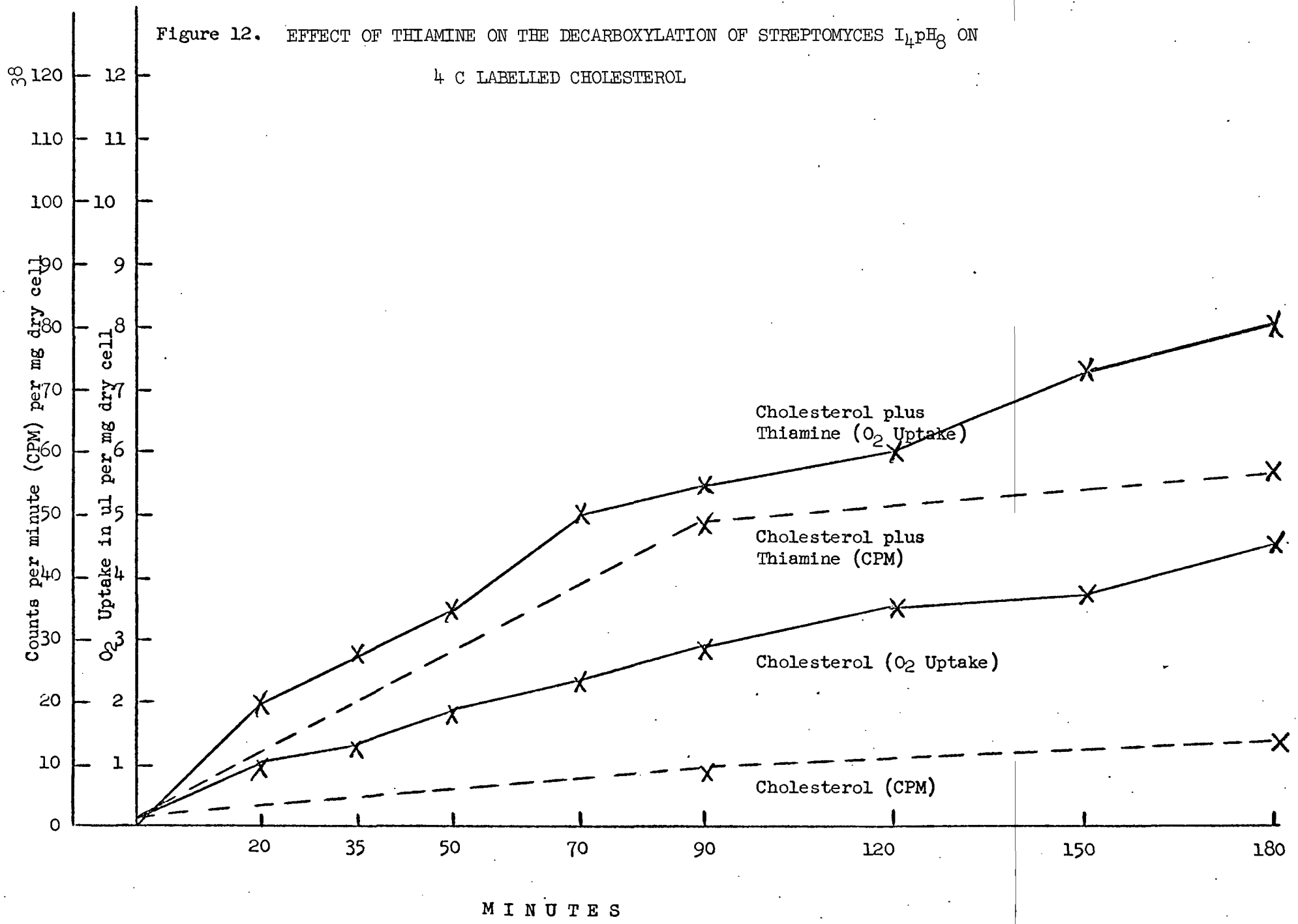
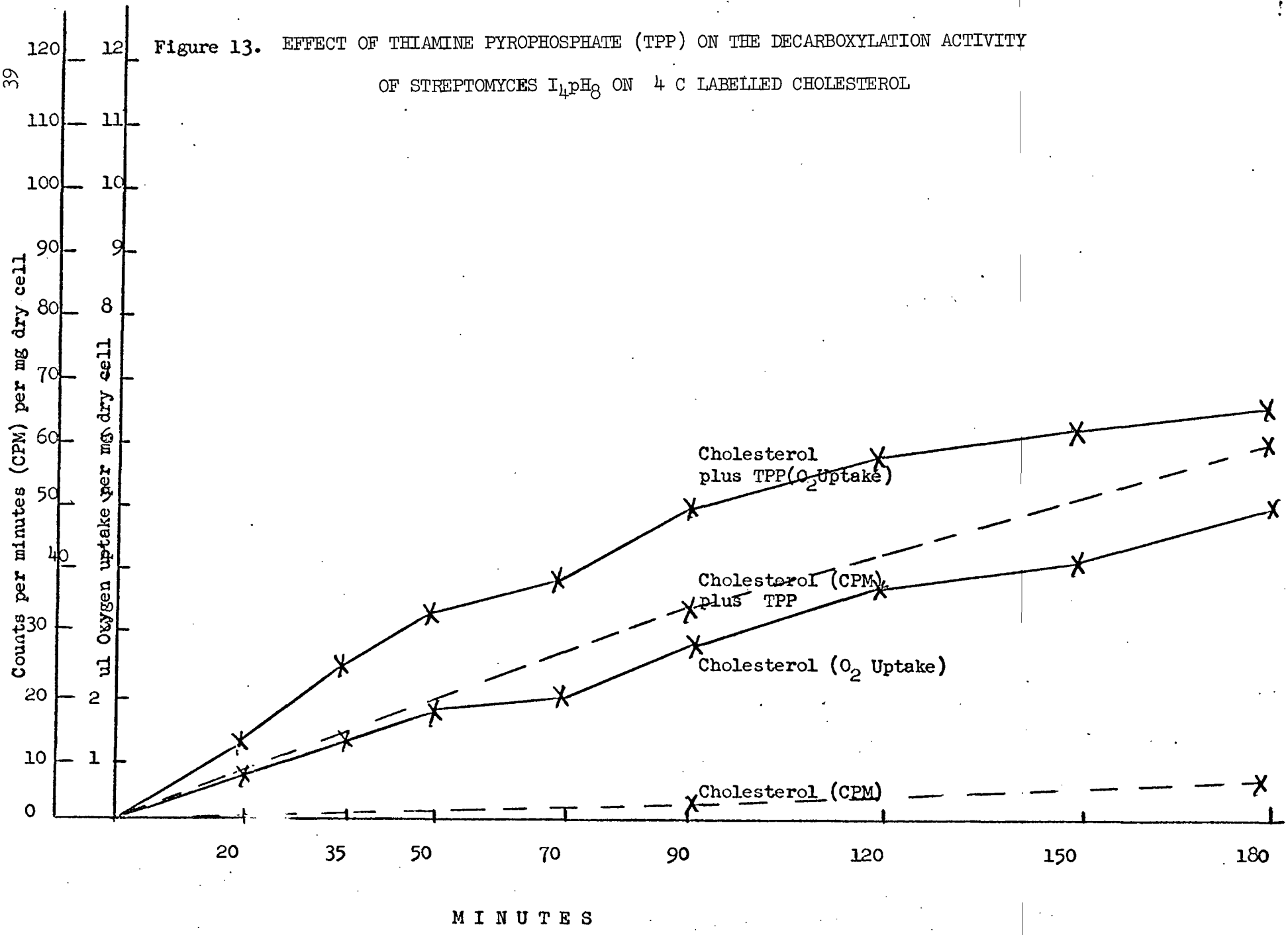


Figure 13. EFFECT OF THIAMINE PYROPHOSPHATE (TPP) ON THE DECARBOXYLATION ACTIVITY OF STREPTOMYCES I_4pH_8 ON 4 C LABELLED CHOLESTEROL



The concentration of thiamine and thiamine pyrophosphate seemed to be important (Fig 14) in affecting cholesterol catabolism. While low concentrations of both thiamine and thiamine pyrophosphate increased the production of radioactive carbon dioxide from cholesterol, too high a concentration (5.0 M mole) seemed to decrease it.

The effect of photolysis of cholesterol under different conditions was studied next. The results are sumarised as in Table 6. A single plus (/) means the detection of two photolysed products (cholestenone and an unidentified product) on the gas chromatograph of cholesterol with 10%but not with a 1% concentration of the sterol in chloroform solution. these spots could not be detected with the less sensitive thin layer chromatographic techniques. Two or three pluses (// or ///) mean the two products were detected in chloroform solutions containing less than 10% of the sterol. In these cases the products could be detected on both the gas chromatogram and the the thin layer chromatograms. The results are tabultaed as qualitative estimates of the amount of cholesterol breakdown products. The best method to store cholesterol seemed to be in a brown bottle at 4 C. Cholesterol appeared to be photolysed faster in a chloroform solution than in the aqueous mineral-salts solution.

The utilization of β-sitosterol by Streptomyces I_4pH_8 was the subject of the next investigation. About 35% substrate utilization was observed in a six-day fermentation period when the inoculum was grown in cholesterol

Figure 14. EFFECT OF DIFFERENT CONCENTRATIONS OF THIAMINE ON OXYGEN UPTAKE BY STREPTOMYCES I_4pH_8

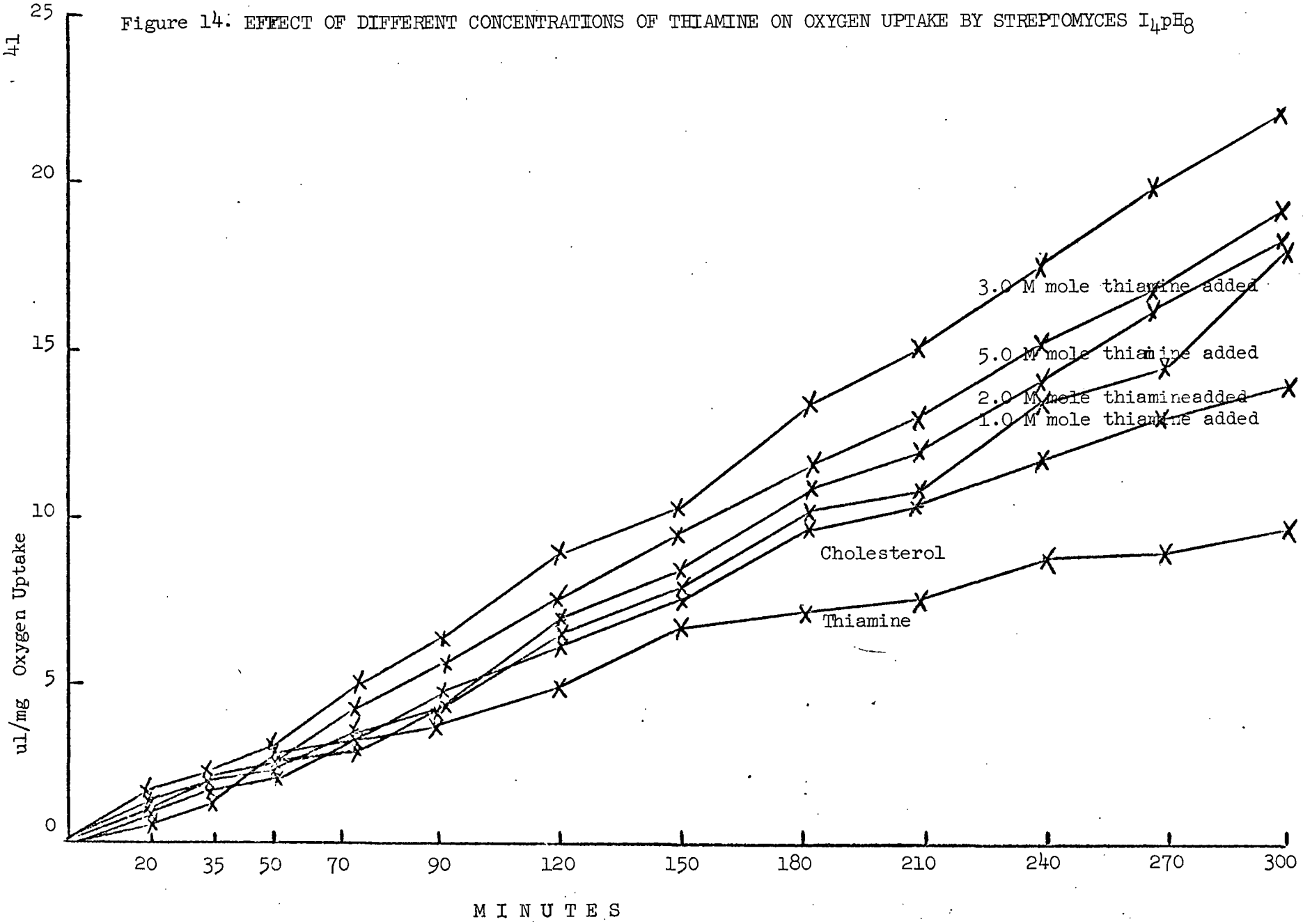


Table 6. Effect of photolysis of cholesterol under different physical conditions

	DAYS					DAYS				
	2	4	6	14	60	2	4	6	14	60
	BROWN BOTTLE					TRANSPARENT BOTTLE				
Laboratory Bench	-	-	-	/	/	/	/	//	///	///
Refreigerator (4 C)	-	-	-	-	-	-	-	/	/	/
Chloroform Layer						-	-	/	//	///
Mineral-salts Solution						-	-	/	/	///

\- No Cholestenone detected

/ // /// Relative amount of Cholestenone detected

mineral salts medium. However when the inoculum Streptomyces I_4pH_8 was prepared by growing in a glucose-mineral salts solution, a higher utilization of β-sitosterol was observed (Fig 15). It was observed that while the maximum utilization of cholesterol is usually over 80% in a six-day period, that of β-sitosterol never exceed 65% in the same period.

On analysis of the β-sitosterol fermentation for intermediates, a spot showed up on thin layer chromatogram at Rf 0.86. This intermediate contained a ketone group and was comparable to that of the standard sitostenone. When this spot was eluted it showed an absorption peak at 240 mu which is typical of absorption properties of the type caused by conjugated double bond systems. The intermediate was eluted and precipitated by digitonin and reverted to its free keto form with pyridine. Both the intermediate and the authentic sitostenone had a melting point of 76 C. The intermediate was thus identified to be the 3-keto derivative of β-sitosterol, sitostenone.

A cell free preparation from a β-sitosterol fermentation flask showed the presence of a β-sitosterol dehydrogenase which converted β-sitosterol into sitostenone (Fig 16).

When β-sitosterol was added to fermentation flasks containing cholesterol, higher dry cell weights and higher rates of both sitosterol and cholesterol utilization were observed. While Streptomyces I_4pH_8 uses about 65% of cholesterol in three days and 40% sitosterol in the same period, this organism used about 78% of the combined substrates in a flask containing 10% sitosterol and 90% cholesterol by volume of a

Figure 15. UTILIZATION OF BETA-SITOSTEROL BY STREPTOMYCES I_4pH_8 CELLS GROWN BY DIFFERENT METHODS

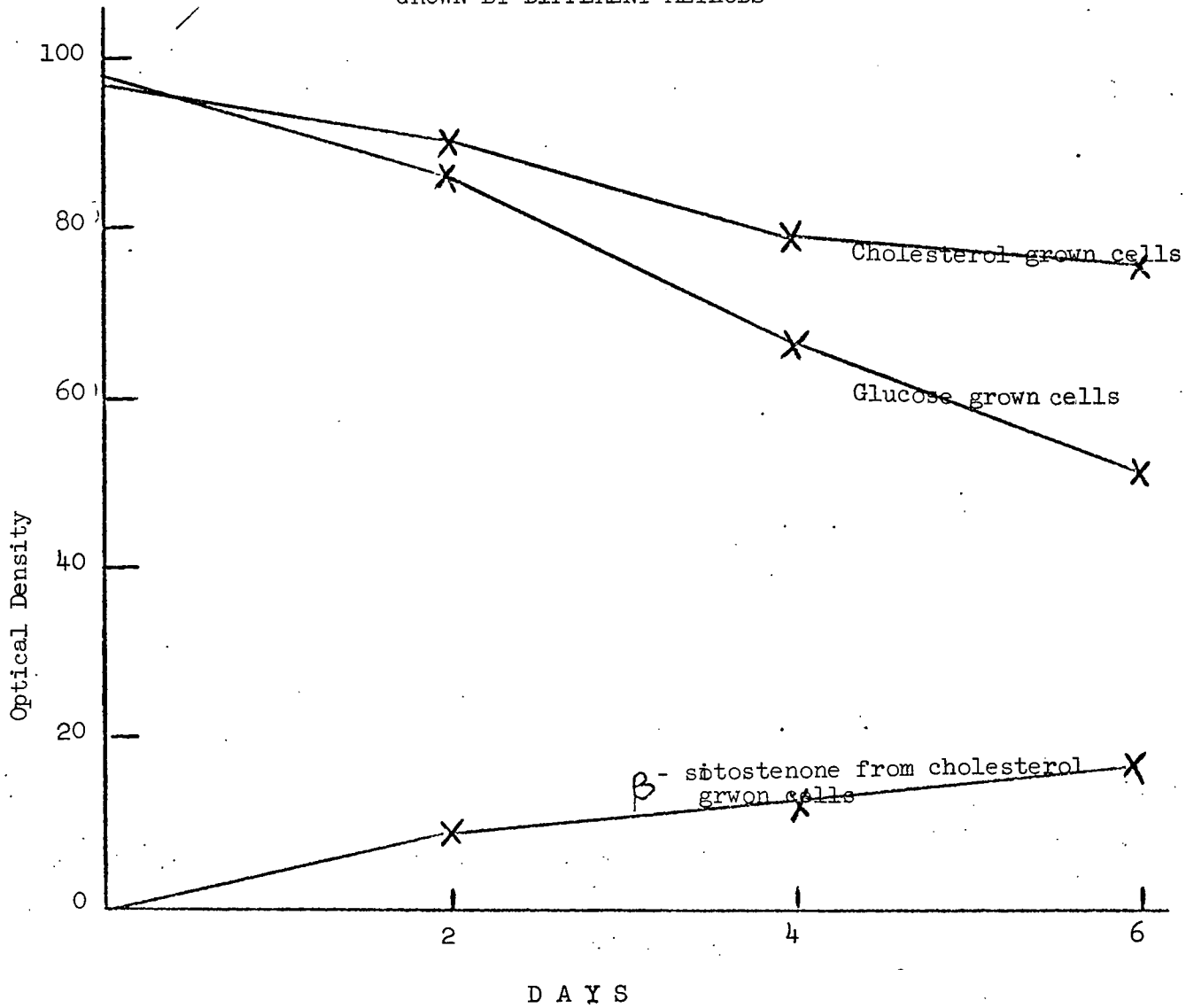
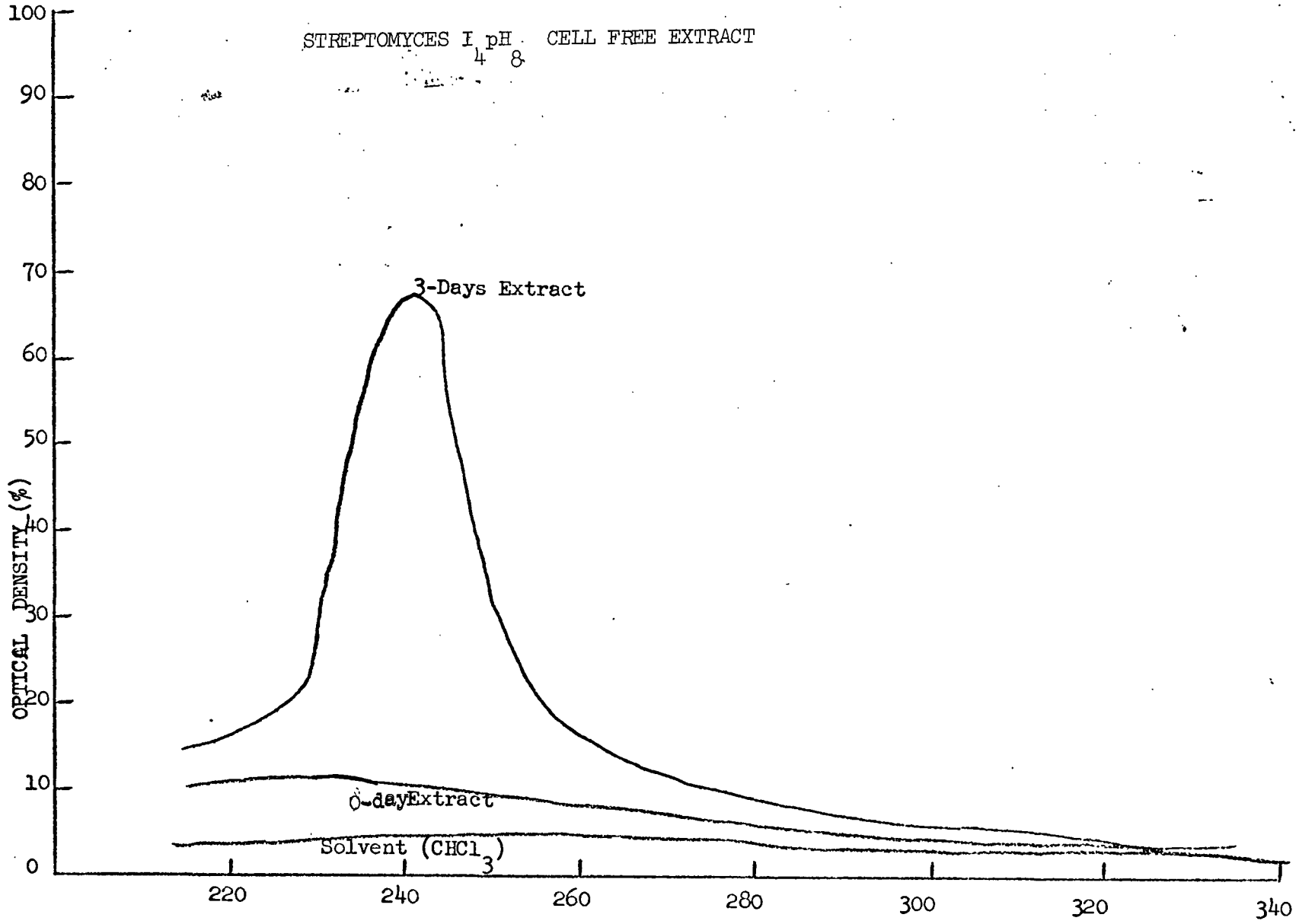


Figure 16. ULTRAVIOLET ABSORPTION SPETRA OF BETA-SITOSTEROL FERMENTATION BY STREPTOMYCES I$_{4}$ pH 8 CELL FREE EXTRACT

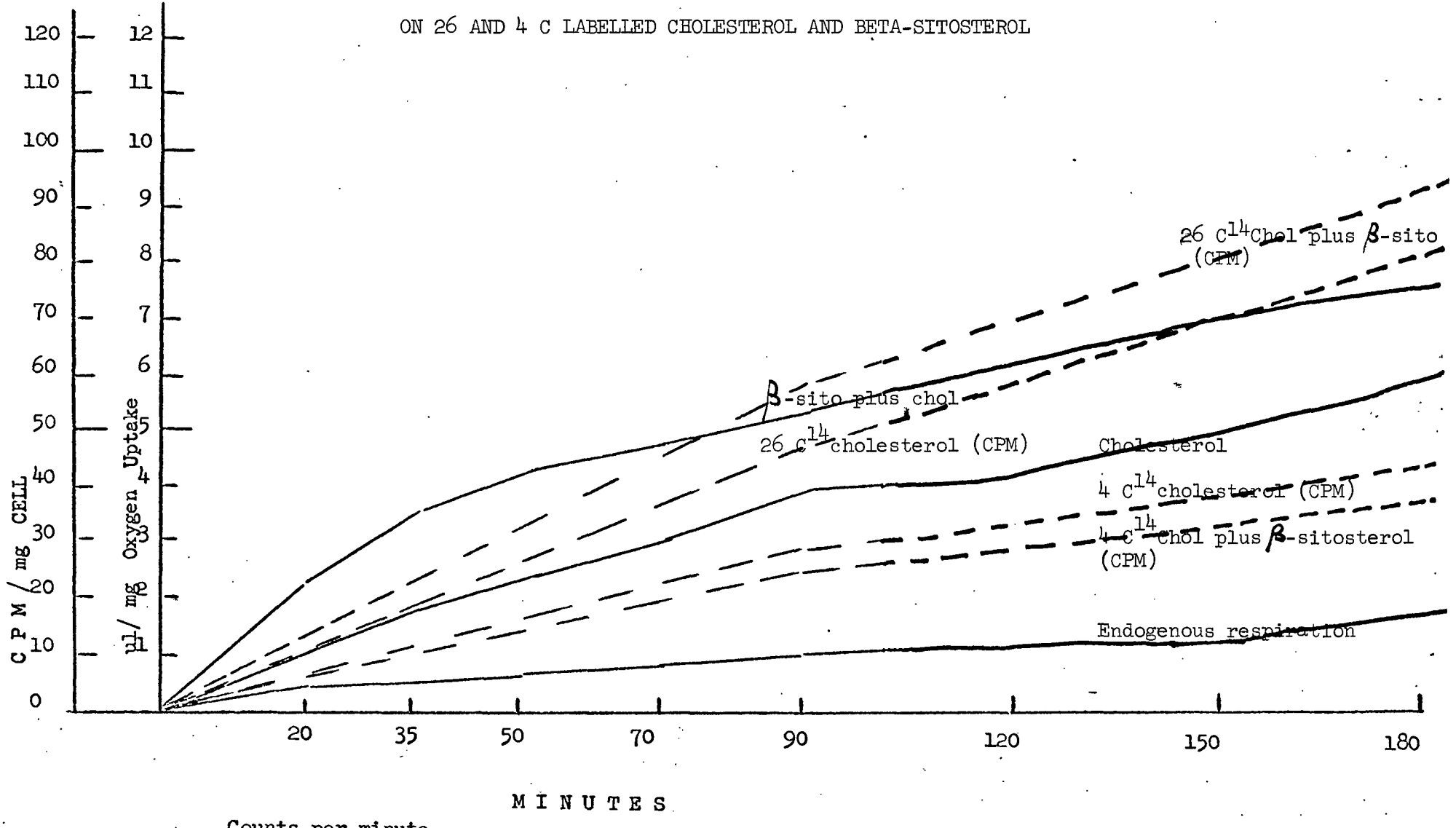


0.1% solution.

A Warburg study using oxygen uptake as a criterion for cell growth also showed the same general result (Fig 17). Since the quantitative determination of β-sitosterol is the same as that used for cholesterol, these experiments did not determined whether the increase of cell growth and oxygen uptake were due to the addition of β-sitosterol to the cholesterol or if there was a higher oxygen uptake and cell growth just because there was more substrate to be used. An experiment using cholesterol labelled at the 4C and the 26C clarified this point. Although there was a higher oxygen uptake in the flask with β-sitosterol plus cholesterol, the $C^{14}O_2$ given off by the cholesterol (in either β-sitosterol plus cholesterol or cholesterol alone) showed no significant difference. The same results were obtained with both the 4C and the 26C labelled cholesterol (Fig 17).

The activities of Streptomyces I_4pH_8 on a varity of steroids were studied. If a certain steriod supported the growth of this organism, this was used as criterion that the organism used the substrate. A sumary of the results of these experiments are in Table 7.

Figure 17. OXYGEN UPTAKE AND COUNTS PER MINUTE (CPM) OF FERMENTATION BY STREPTOMYCES I_4pH_8 ON 26 AND 4 C LABELLED CHOLESTEROL AND BETA-SITOSTEROL



Counts per minute;
Oxygen Uptake

Table 7. Growth of Streptomyces I_4pH_8 on various sterol compounds

COMPOUND	Growth of Organism
Cholesterol	Positive
Dihydrocholesterol	Positive
Δ^4-Cholestene-3-one	Positive
Δ^5-Cholestene-3-one	Positive
Cholestan-3-one	Positive
Cholic Acid	Negative
Progesterone	Positive
Pregnenolone	Positive
4-Androstene-3,17dione	Positive
Esterone	Positive
Stigmasterol	Positive
4, 22 Stigmastidiene	Positive
3-oxobisnor-4Cholen-22ol	Positive
βSitosterol	Positive
Sitostenone	Positive
Desoxycholesterol	Positive
Δ^4-Cholesten-4ol-3-one	Negative

V. DISCUSSION

Lewis (1962) studied the effects of various nitrogen sources on cholesterol degradation by Streptomyces sp. in a cholesterol-mineral salts solution. Using NH_4NO_3 as the only nitrogen source, Lewis found that at the end of a six-day period, some 62% of the cholesterol was utilized. In another experiment using $(NH_4)_2SO_4$ as the nitrogen source, about 46% of the cholesterol was degradated at the end of the same period. The initial pH value in both experiment were about 7.0, but they dropped to about 3.5 after 6 days of growth. In still another experiment, using KNO_3 as the nitrogen source some 80% of the cholesterol was utilized at the end of a six-day period. The pH started at 7.0 in this experiment too but went up to about 8.5.

Turfitt (1946) also did some submerged culture studies with his isolates using various pH values and noted the same results as those obtained by Lewis. He postulated that the drop in pH was due to the metabolism of both the ammonium ions and the cholesterol. Lewis believed that when the ammonium radical was utilized hydrogen ions were released which gave raise to the acidity of the medium. When KNO_3 is used as the nitrogen source, although the NO_3^- may be converted to NO_2^- then to NH_4^+ radicals and the same accumulation of hydrogen ions might occur, Lewis postulated that for every NO_3^- radical degradated a potassium ion is also released. The chemical nature of the potassium ion, as a proton acceptor helps to neutralize the effect of the acid and keep the pH constant. Of course the drop of the pH

might be due to the accumulation of organic acids resulted from the metabolism of the cholesterol molecule itself, but there is very little evidence of significant concentration of organic acids during cholesterol catabolism.

These experiments first duplicated the results of Lewis using Streptomyces I_4pH_8. Four different nitrogen sources were used and the pH changes, substrate utilizations etc. were observed carefully. The results which agreed with those of lewis are shown on Fig 1 & 2. In another experiment using KNO_3 as the sole nitrogen source, the initial pH value were adjusted through a range of 5 to 10. It was observed in this experiment that after three days the pH of all the cultures had changed to between 8 and 9 and they stayed there throughout the experimental period (Fig 3). The dry weights and the cholesterol utilized during this experimental period are shown on Fig 4. These values are expressed in mg/ml. Near pH was a maximum utilization of cholesterol and a maximum production of cells.
A different mineral medium was used in another experiment. The M-9 synthetic medium consists of two solutions which have to be autoclaved separately and mixed together after cooling. It has a much better buffering capacity than the cholesterol-mineral salts solution due to the presence of two phosphate salts. The nitrogen source used here is NH_4Cl. Fig 5 shows a comparision of the 2 media. It was noted that maxium dry cell weights and cholesterol utilizations were obtained at pH 8 in both cases. But the M-9 synthetic medium showed an appreciably higher dry cell weight and substrate utilization than that of the mineral salts medium at the end of three days.

The results of these experiments may be explained by the fact that the M-9 synthetic medium has a better buffering capacity and thus keeps the organism growing at an optimum pH 8 throughout the experiment. Thus the nitrogen source , whether it be an ammonium salt or KNO_3, really does not have much effect on the utilization of cholesterol. These results agree too with reports in literature that Streptomyces sp. in general have an optimum pH for growth at 8.0-8.5.

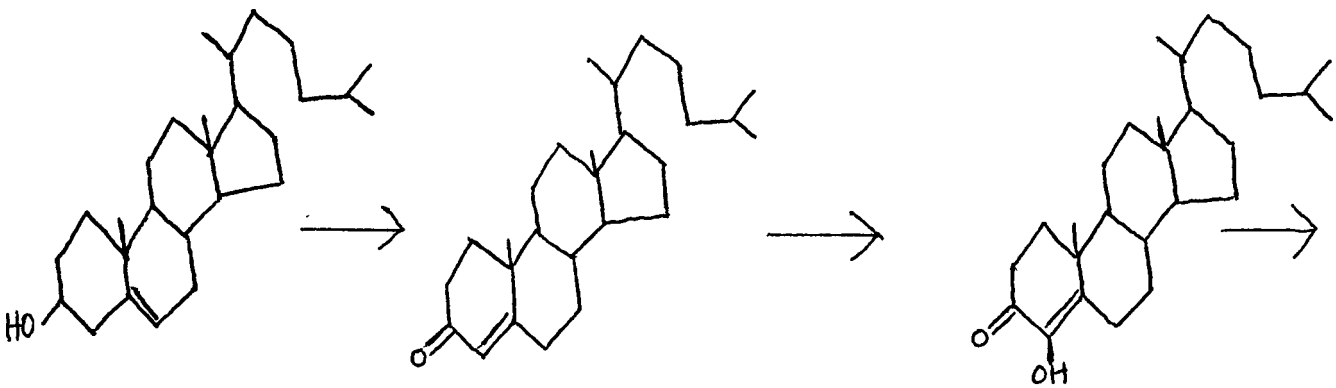
At least two points of attzck on the cholesterol molecule have been demonstrated conclusively. Stadtman et al (1954), using an organism Schatz (1949) isolated, didi radioisotope studies with $4C^{14}$ and $26C^{14}$ labelled cholesterol. She noted that the $4C^{14}$ was decarboxylated about 4 times as fast as that of the $26C^{14}$ of the cholesterol molecule. Peterson and Davis (1964) did similar studies with Streptomyces I_4pH_8 and noted that the 26C was decarboxylated twice as fast as that of the 4C of the cholesterol molecule.

In the Warburg manometer studies sumarized in Fig 10-13, the decarboxylation of cholesterol was again shown to be about twice as fast with the 26C than with thw 4C of the cholesterol molecule. Thiamine or thiamine pyrophosphate did not affect this relationship. Thus, if these factors are required for decarboxylation, it would seem that are required to the same extent for the removal of both the no. 4 and the no. 26 carbon atoms.

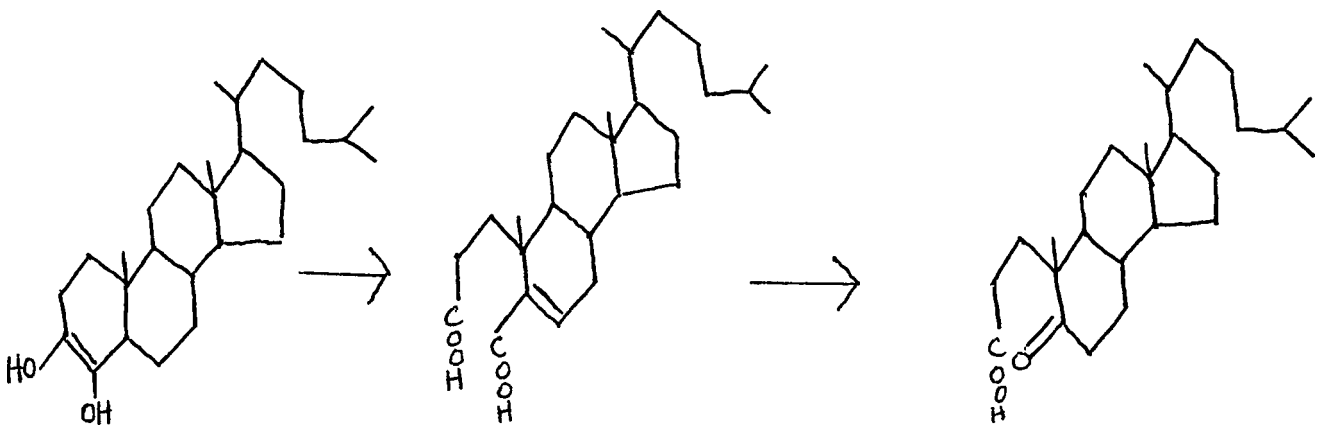
A perusal of the literature shows that though some possible intermediate products have been isolated during the microbial degradation

of cholesterol, there is little known about the mechansim of removal of no. 4 carbon and no. 26 carbon.

A 4 carbon degradatation as suggested by Brown (1964) is as follows:

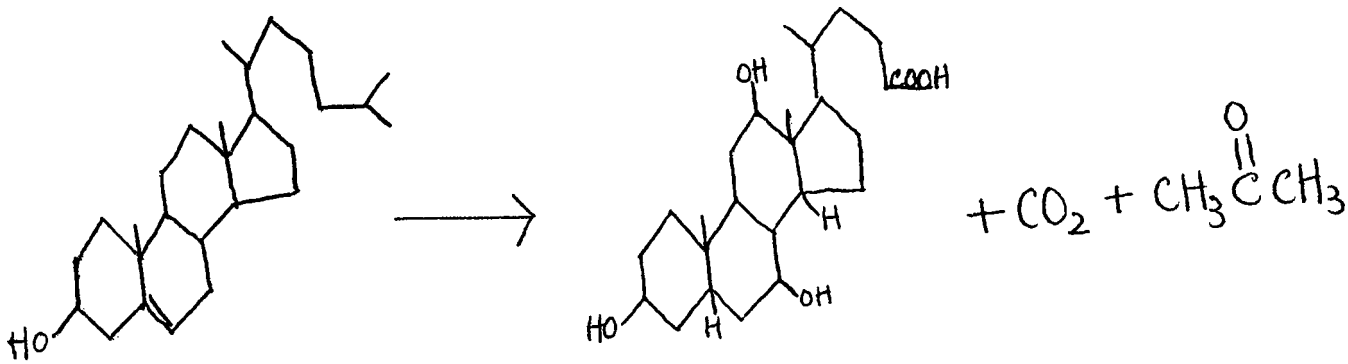


Cholesterol — Δ 4-Cholestene-3one — Δ 4 Cholestene-4ol-3one (Brown 1964)



-enol form — Diel's acid — Windau's keto acid (Turfitt 1948)

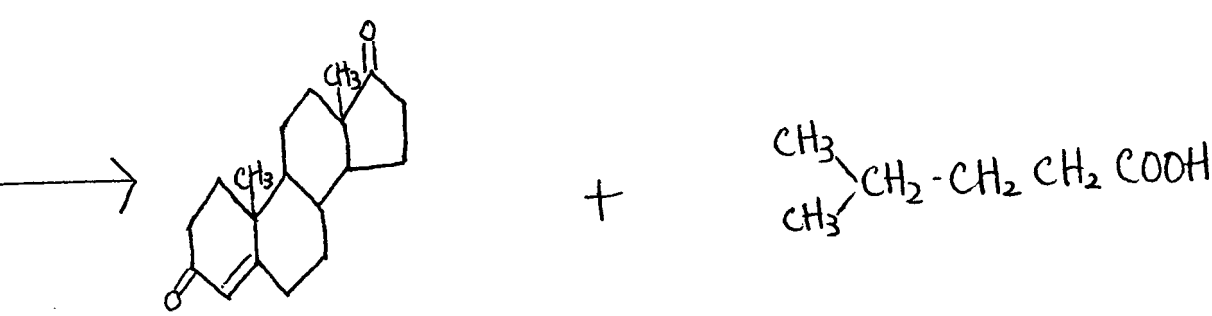
Evan less is known about the removal of the 26 carbon. Among important intermediates, cholic acid was identified by Davis (1962), Δ1,4 androstadiene-3-7-dione by Whitmarch (1964), isocaproic acid by Turfitt (1948), and methylpheptanone by Kramli and Horvath (1947). It seems likely that the removal of the 25 carbon may include these intermediate compounds. If such a process occurs at the side chain, one molecule each of cholic acid, CO_2 and acetate would be formed from a molecule of cholesterol. This process would provide energy for a direct attack on the steroid molecule. One might propose this scheme to occur as follows:



Δ4Cholestene-3one

Cholesterol

Cholic acid
(Davis 1962)



Δ1,4 androstadiene-
3, 17-dione

(Whitmarch 1964)

Isocaproic acid (Turfitt 1948)
or Methyl-heptanone

As far as is known all thiamine pyrophosphate requiring enzymes catalyse either decarboxylation of α-keto acids or cleavage of α-hydroxyl-ketoned or α-diketones. A similar mechanistic problem exists in each of these enzymic reactions and thiamine pyrophosphate is a special reagent which is necessary to solve this problem. In each case of a thiamine pyrophosphate reaction, a bond is broken immediately adjacent to the carboxyl group. In each case the over all reaction is a removal of a group R (or RH) and replacement by another group R'. The R groups are all of such structure that they can leave as stable compounds (plus hydrogen ions) if the bonding to be broken is cleaved by withdrawal of the bonding electron pair toward the carboxyl group and into the thiamine-substrate enzyme complex.

While the thiamine pyrophosphate mechanism may be used to explained the proposed mechanism of the decarboxylation of the 26 carbon (most probly involving α-keto compounds resulting from cleavage of the side chain), no such immediate explaination can be applied to that of the 4 carbon. Of course, much of this may be due to the incomplete knowledge of what is going on during the degradation of the cholesterol molecule. Nevertheless, in these experiments thiamine pyrophosphate did not exert a different effect

on the removal of the 4 carbon and the 26 carbon. Although the effect of thiamine pyrophosphate is masked by the fact that it can be used as a sole carbon source to a certain extent (Fig 9), there was sufficient proof that both thiamine and thiamine pyrophosphate do effect the decarboxylation of the cholesterol molecule. This was shown not only in the manometric experiments, but both thimaine and thiamine pyrophosphate in low concentrations increased the dry cell weights and cholesterol utilization in fermentation flasks also.

It was shown clearly (Table 5) that a periodic transfer of the cholesterol-grown cells back to a glucose-mineral salts solution greatly enhanced the growth of the organism. This might be explained by Davis' theory of an adaptive enzyme present in this particular organism Streptomyces I_4pH_8. Peterson and Davis (1964) showed that glucose-grown cells showed a stimulation in degradation cholesterol by Streptomyces I_4pH_8 only after an initial lag of 1 hr. In contrast, cells grown in cholesterol showed no lag in oxygen uptake during catabolism of cholesterol. They concluded that the enzymes involved in cholesterol metabolism in this particular organism is of an inductive nature.

The period of starvation is also critical (Fig 8) as too little starvation leads to the 'carrying over' of adsorbed cholesterol and too much starvation leads to autolysis of the cells. In either case manometric experiments cannot be carried out efficiently. The ideal cell weight for inoculum has a population of 14 mg per ml after being starved for 36 hr prior to the experiment.

Bergstrom, Wintersteiner (1942), Neher (1958), Hais and Masters (1964) are investigators that have studied the photolysis of cholesterol. Hais and Masters noted that photolysis of cholesterol takes place in dilute aqueous solution as well as in dry state on glass or filter paper. However, they also noted that photolysis was negligible if precautions were taken. Experiments done during this study showed that when stored in a brown bottle the photolysis is negligible after 14 days on the laboratory bench. Only a sterol solution in chloroform of more than 10% concentration showed impurities on the gas chromatogram. Other less sensitive methods such as thin layer chromatography and spectrophotometry failed to show such impurities. The sample taken at 60 days showed essentially the same results. However, the chloroform and mineral-salts solution flasks stored on the shaker showed a more rapid rate of photolysis (Table 6). Spots of impurities on thin layer chromatogram as well as on the spectrophotometer appeared after a 14-day period. These impurities are typical of that of cholestenone. However as the dyration of all experiments in fermentation flasks rarely exceeded six days, photolysis was perhaps not a major source of error in the various identification of intermediates from the microbial degradation of cholesterol.

β-sitosterol is a plant sterol very similar to cholesterol. The ability of Streptomyces I_4pH_8 to use β-sitosterol was demonstrated (Fig 15). The ability to use β-sitosterol as a sole carbon source can be enhanced by growing the inoculum in a glucose-mineral salts solution (Fig 15).

The ability of the organism to use this plant steroid was never as efficient as its ability to use cholesterol. This may be due to the presence of a slightly more branched side chain as this is the only difference between this steriod and cholesterol.

A cell free "β-sitosterol dehydrogenase" has also been found which can convert β-sitosterol to sitostenone. Thus the degradation processes of both cholesterol and the plant sterol have very much in common.

The effect of sitosterol on the metabolism of mammalian cholesterol had been studied by many investigators. Using rats and radioisotope techniques, Gerson and Shortland (1964) found that the presence of sitosterol can induce a higher rate of overturn of bound cholesterol which leads to a rapid rate of metabolism of cholesterol. In these studies, a 10% sitosterol solution was added to a cholesterol fermentation flask and an increase in both dry cell weight and substrate utilization over that of either cholesterol or sitosterol alone was observed. However, since the quantitative determination of both cholesterol and sitosterol used in these studies were the same (Lieberman-Burchard method), a more detailed experiment using radioactive cholesterol was made (Fig 17). While the mixture of sitosterol and cholesterol did showed a higher oxygen uptake than that of either cholesterol or sitosterol alone, the radioactivity of the sitosterol and cholesterol mixture showed no significant difference using either 4 or 6 carbon labelled cholesterol (Fig 17). These results seem to favor the interpretation that there is a higher growth rate in the cholesterol-sitosterol mixture just because

there are present two kinds of substrate this particular organism can use. The addition of sitosterol to a cholesterol medium does not seem to affect the rate of cholesterol metabolism in Streptomyces sp.

A number of steriods can be used as the only source of carbon by this organism (Table 7) using growth as the only criterion. As to why this organism cannot grow in cholic acid and Δ4-cholesten-4ol-3one as the sole carbon source is not known since these steroids had been identified as intermediates of the degradation of cholesterol. A low pH cannot be attributed to as a reason for this failure to grow as the pH of these experiments were very closely observed. It is possible that permeability factors could be involved, although this cannot be checked.

IV SUMMARY

The study of the effect of the various nitrogen sources on cholesterol degradation by Streptomyces I_4pH_8, was carried out. Experiments were also performed at different pH values and with different buffer solutions. These results were correlated with those of the nitrogen sources, and it was indicated that a pH of 8.5 gave the optimum growth rate with this organism regardless of the nitrogen source used.

Thiamine and thiamine pyrophosphate were found to enhance the removal of both the 4 carbon and the 26 carbon of the cholesterol molecule to about the same degree.

Streptomyces I_4pH_8 was found to use β-sitosterol as a sole source of carbon. The pathway of degradation of β-sitosterol seems to follow closely that of cholesterol. An intermediate sitostenone was identified.

The presence of β-sitosterol in cholesterol medium did not appear to increase the rate of cholesterol degradation. But, this extra carbon source did increase the growth rate of the organism.

Anumber of steroids were found to be able to serve as the sole carbon source for the growth of Streptomyces I_4pH_8 but two intermediates of cholesterol degradation, cholic acid and Δ4-cholesten-4ol-3one, did not support growth of the organism.

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