and in returning to the ground state be reconverted to DBA^{++} itself. An oxygenated derivative of DBA^{++} is presumed to be the emitting species, since the luminescent light is blue whereas the fluorescence of DBA^{++} is green. Since available evidence indicates that enzymically generated $O_2^-\cdot$ remains bound to the enzyme surface, and since no light emission was observed when DBA^{++} reduction and $O_2^-\cdot$ production occurred on separate enzymes, the chemiluminescent reaction is believed to take place on the enzyme surface.

If light emission is a consequence of the interaction of DBA° or $DBA^{+}\cdot$ and an oxygen radical, and if the production of each of these is dependent, in turn, upon substrate concentration, then it should follow that the intensity of light emission varies, in the usual way, with the square of the substrate concentration. Hence, there appears to be no need to postulate (Totter *et al.*, 1960a) either two substrate binding sites on xanthine oxidase or an obligatory four equivalent reduction of DBA^{++}. The observation that luminescence is directly proportional to enzyme concentration is not in conflict with these considerations if the DBA° or $DBA^{+}\cdot$ and $O_2^-\cdot$ interact on the enzyme surface, at, or close to, their point of generation, rather than in free solution. Finally, it should be noted that the low concentrations of H_2O_2 generated during the aerobic action of the enzyme could account for less than $^1/_{1000}$ of the intensity of enzyme-induced luminescence. Indeed, the addition of 10^{-4} M H_2O_2 has no perceptible effect upon the time course of luminescence. Thus, the role of H_2O_2 in the enzymic luminescence appears to be negligible.

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Effect of Metrazol on Isolated Mammalian Cells. II. Inhibition of Synthesis of Cholesterol*

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Cultures of strain L mouse fibroblasts grown in the presence of a metabolic stimulant, Metrazol (pentamethylene tetrazol), had a lower sterol content than control cultures. Cells incubated in the presence of the drug with acetate-1-C^{14} incorporated one-third of the radioactivity normally incorporated by control cells. Cells grown in the presence of Metrazol but incubated in Metrazol-free media rapidly synthesized sterols of high specific activity. Rigorous analysis of the sterol fraction indicated that 78% of the radioactivity was present in cholesterol.

Cultured mammalian cells accumulate lipids in the lag phase following cessation of the logarithmic phase of growth (King *et al.*, 1959; Bensch *et al.*, 1961). This lipid accumulation decreases gradually as old lipid-laden cells are induced to multiply. The rate of cholesterol synthesis in cultured cells, whether L strain mouse fibroblasts or Ehrlich ascites cells, increases when protein synthesis is inhibited. These results suggested

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to us that an increased rate of growth might be accompanied by reduced cholesterol synthesis.

We described previously the enhanced growth rate of L strain cells in the presence of Metrazol (pentamethylene tetrazol) (Alexander and Alexander, 1961a). Addition of this drug also results in increased oxygen uptake by cell cultures, but this increase is not as pronounced as the increase in growth rate. The over-all effect of addition of Metrazol, therefore, is a decrease in oxygen uptake per cell. This parallels the data obtained in *in vivo* studies of Metrazol-induced convulsive seizures which bring about a localized hypoxia while increasing oxygen uptake by the brain (Gourdjian *et al.*, 1946).

In this study, we have endeavored to determine whether Metrazol inhibits synthesis and accumulation of cholesterol in mouse fibroblasts.

Materials and Methods

Mouse fibroblasts, strain L, NCTC 929, obtained from Microbiological Associates, Bethesda, Md., were maintained and incubated in minimum essential medium (Eagle, 1959) supplemented with 10% horse serum and a penicillin-streptomycin mixture, all of which were purchased from Microbiological Associates. Metrazol was obtained from the Bilhuber-Knoll Corporation, Orange, N. J. Cells were grown in monolayer cultures and in a perfusion chamber as described before (Alexander and Alexander, 1961a). Monolayer cultures were routinely grown in culture flasks with and without Metrazol. They were fed twice a week and transferred weekly. The cells were scraped with a rubber policeman, harvested, divided into two equal portions, and placed in fresh flasks. The Metrazol level, at each feeding or transfer, was maintained at 4×10^{-4} M. All flasks, whether with Metrazol added to the medium or not, were handled in exactly the same manner. Duplicate cultures out of a large number were withdrawn from circulation and the cells were washed with saline, harvested, washed again with centrifugation, and either resuspended for incubation with labeled acetate or dried for weighing in an oven at 110°. Incubations were carried out in 25-ml Erlenmeyer flasks with swirling on a rotary table to prevent the cells from settling and to increase aeration.

A large inoculum from monolayer cultures was transferred to a U-shaped chamber and the cells grown in submerged, strongly aerated culture with continuous inflow of fresh medium and outflow of spent broth. Vigorous aeration kept the cells in suspension and prevented adhesion to glass surfaces. Aliquots were withdrawn at fixed intervals and viable cells were counted in a hemocytometer. Metrazol was added to a culture in a logarithmic phase of growth, both to the incubation chamber and to the reservoir of fresh medium. Eighty hours after addition of the drug a 20-ml portion of the culture was withdrawn, centrifuged, washed exhaustively with isotonic saline, resuspended in Metrazol-free nutrient medium, and distributed in equal portions into 10 Erlenmeyer flasks. Each flask received 1.0 μc of acetate-1-C^{14}. Five flasks received also 1.2 mg of Metrazol each, thus restoring in them, except for the radioacetate, the original conditions existing in the culture prior to harvesting of cells and "washout" of the drug. The other 5 flasks contained cultures of cells grown in the presence of Metrazol but suspended in Metrazol-free medium. All 10 flasks were incubated for 3 hours with swirling.

Total sterol content was determined with Liebermann-Burchard reagent after hydrolysis of the washed cells, removal of fatty acids, and precipitation of sterols with digitonin (Sperry and Webb, 1950). The cultures did not contain enough material to permit a detailed examination of the sterol fraction, and it was impractical to grow enough cells to obtain a sufficient amount of sterols. Therefore, cultures were incubated with acetate-1-C^{14}, so that cholesterol-C^{14} synthesized in the cells could be diluted with carrier cholesterol prior to purification. Cholesterol digitonides were decomposed with pyridine. The resulting free sterols were weighed, recrystallized to constant specific activity, and plated for radioassay in a Q-gas Geiger counter. The sterol fractions synthesized by washed cells from perfused cultures were purified further after addition of 10 mg of carrier cholesterol. Purification steps involved repeated crystallizations from ether-ethanol and aqueous ethanol, precipitation of cholesterol dibromide (Schwenk and Werthessen, 1952), reduction of the dibromide with powdered zinc, and final recrystallization of cholesterol to constant specific activity.

Results

Growth Patterns of L Cells in the Presence of Metrazol.—Addition of Metrazol to a perfused culture caused a sharp drop in the number of viable cells, similar to the one reported by us previously (Alexander and Alexander, 1961a). The number of cells decreased from 8×10^8 per ml to 1×10^8 in 30 hours. Afterwards the number of cells began to increase, reaching the logarithmic phase of growth in approximately 20 hours. The number of cells grew very rapidly thereafter, necessitating increasingly frequent withdrawals of cells from the culture chamber. The generation time, which never decreased below 80 hours in control runs, went down to 9 hours in a typical experiment (Table I).

An adaptation period was observed after addition of Metrazol to monolayer cultures. During the first 3 days growth was negligible, but afterwards the cells began to multiply rapidly. In the 4-week period between the first and the fifth transfer, control cells multiplied 41 times (from an inoculum containing 0.72 mg of cells to 16 flasks, each containing 1.86 mg of cells). Metrazol-treated cells multiplied 107 times, from an inocu-

TABLE I

GROWTH OF CELLS IN PERFUSED CULTURES

All cultures were in the lag phase of growth. Cell count began 80 hours after addition of Metrazol, to allow for the adaptation period of normal cells to Metrazol (see text).

Culture	Mean Cell Count × 10^6								Optimum Generation Time (hr.)
	interval (hr.)								
	0	1	2	3	4	5.5	6	7	
Control	506	511	516	520	526	—	—	545	84
Metrazol a	681	701	728	766	819	946	—		11
Metrazol b	816	849	891	946	1019	—	1145		9.1

TABLE II

GROWTH AND STEROL CONTENT OF MONOLAYER CULTURES

During the adaptation period after addition of Metrazol the cultures remained stationary; therefore, measurements during the first week are not indicative of the true rate of growth of Metrazol-adapted cells.

Culture	Growth Period				
	1 Week		3 Weeks		5 Weeks
	Wt. of Cells (mg)	Wt. of Sterols per mg Cells (μg)	Wt. of Cells (mg)	Wt. of Sterols per mg Cells (μg)	Wt. of Cells (mg)
Control	1.43	30.1	5.92	32.1	29.76
Metrazol	1.11	15.7	9.52	14.0	59.04

lum containing 0.55 mg to 16 flasks, each with 3.69 mg of cells (Table II).

Sterol Metabolism of Strain L Cells in Monolayer Cultures.—Addition of Metrazol to monolayer cultures caused a striking decrease in the sterol content of cells (Table II). One week after addition of the drug the number of cells in Metrazol-treated cultures was still somewhat lower than the number in control cultures, because of the initial lag. After 3 weeks, the number of cells was 61% higher. The sterol level was nearly halved in 1 week and remained near 50% in treated cultures as compared with untreated ones.

Harvested monolayer cells resuspended and incubated with acetate-1-C^{14} incorporated a small but definite amount of radioactivity into sterols even in the presence of cholesterol in the medium (Table III). Similar cells grown and incubated in the presence of Metrazol incorporated a significantly smaller amount of radioactivity into sterols. Even though there were more cells in Metrazol-treated cultures, the total recovery of radioactivity in the sterol fraction was only 36% of the comparable total in control cultures. Specific recovery per mg of Metrazol-treated cells was only 22% of the recovery per mg of control cells.

Sterol Synthesis in Cells Grown in the Presence of Metrazol After the Drug Was Washed Out.—

TABLE III

INCORPORATION OF ACETATE-1-C^{14} INTO STEROLS

Cells were subcultured three times. Metrazol concentration was maintained at 4×10^{-4} M at each feeding. Harvested control or Metrazol-treated cells were washed and resuspended in 10 ml of respective media in five 25-ml Erlenmeyer flasks. After a 3-hour incubation with 1 μc of acetate-1-C^{14} per flask, cells were harvested and analyzed.

Culture	Total Recovery of C^{14} in the Sterol Fraction (cpm)	Incorporation of C^{14} into Sterols per mg of Cells per Hour (cpm)
Control	4,205	934
Metrazol	1,493	208

Eighty hours after addition of the drug, when the cells for this experiment were withdrawn from the culture chamber, they showed little sterol synthesis when incubated in a medium containing Metrazol. However, washed cells incubated in a Metrazol-free medium synthesized sterols with unusual rapidity (Table IV). Presence of Metrazol during growth apparently suppressed synthesis or accumulation of sterols in these cells

TABLE IV

SYNTHESIS OF CHOLESTEROL BY CELLS, GROWN IN THE PRESENCE OF METRAZOL, INCUBATED IN METRAZOL-FREE MEDIA

Cells were grown in perfusion culture in the presence of 4×10^{-4} M Metrazol, harvested, washed thoroughly with isotonic saline, then suspended in 20 ml medium and divided into 10 Erlenmeyer flasks. Five flasks received Metrazol. Each flask received 1 μc of acetate-1-C^{14}. After a 3-hour incubation, the contents of each batch of five flasks were pooled for analysis.

Culture	Dry Wt. of Cells (mg)	C^{14} Found in the Sterol Fraction (cpm × 10^3)
Washed cells, suspended in Metrazol-free medium	10.84	660
Washed cells resuspended in medium containing 4×10^{-4} M Metrazol	10.84	8.9

TABLE V
PURIFICATION OF CHOLESTEROL-C^{14} SYNTHESIZED BY STRAIN L CELLS

	Metrazol-Free Culture (cpm/mg)	Culture Containing 4×10^{-4} M Metrazol (cpm/mg)
Digitonin-precipitable sterols[a]	1,535,000	—
Sterols after dilution with carrier cholesterol	61,630	448
Sterols after 3 crystallizations	51,522	389
Cholesterol purified through the dibromo derivative[b]	48,297	328

[a] Sterol fractions obtained in experiment described in Table IV. Since the amount of sterols in cells incubated in the presence of Metrazol prior to addition of carrier could not be determined no specific activity can be given (however, see Table IV for total radioactivity). [b] Purification through dibromide was carried out as described by Schwenk and Werthessen (1952).

but did not alter them permanently. Removal of the drug from the environment enabled these cells to compensate for the deficiency in their sterol content at a rapid rate. Purification of the sterol fraction through the dibromide brought the specific activity of the resulting cholesterol to 78% of the starting value (Table V). The specific activity of sterols synthesized in the presence of Metrazol and treated in the same manner decreased to 73%. Thus, three-fourths of the radioactivity in the digitonin-precipitable sterol fraction was due to cholesterol itself and not to "high counting companions" (HCC) described by Schwenk *et al.* (1955).

DISCUSSION

The amount of cholesterol present in the cells at any given time is a result of the interplay between cell metabolism and the exchange of metabolites between the cell and the medium. Recovery of radioactivity in the sterol fraction reflects the uptake of acetate-1-C^{14} by the cell, conversion to cholesterol-C^{14}, excretion, and degradation to other metabolites. Decreased conversion to cholesterol-C^{14} may be due to diversion of acetate-1-C^{14} to other uses within the cell or to blockage along the acetate-cholesterol pathway. Recovery of cholesterol-C^{14} is not a measure of utilization of acetate in sterol synthesis, since some of the radiocholesterol has been lost through degradation to other metabolites and some through excretion into the medium. It is, however, an indication that such utilization has indeed taken place and a measure of the minimal amount that must have been synthesized in the cells.

Utilization of acetate-1-C^{14} in sterol synthesis was estimated on the basis of total radioactivity residing in cholesterol rather than of specific activity. In this way we have eliminated the effect of the size of the pool present. Specific radioactivity per unit weight was used only in the purification procedure described in Table V, which showed that the radioactivity is indeed associated with cholesterol.

To promote rapid growth and multiplication of cells, we considered it necessary to add 10% horse serum to our media. The parent cells, *in situ*, also exist in an environment containing serum; whether they synthesize sterols in the presence of serum cholesterol is not known. They may have the ability to do so when the level of sterols in the environment is decreased sharply. Under our experimental conditions, fibroblasts existing in a medium containing a very low level of cholesterol never exceeding 8 mg per 100 ml synthesized sterols as evidenced by the incorporation of acetate-1-C^{14} into cholesterol. In the experiments of Bailey *et al.* (1959) with MB III mouse lymphoblasts, even in the presence of 86–93 mg of cholesterol per 100 ml of medium 5% of the total cell cholesterol was derived from synthesis within the cells. Synthesis of cholesterol from acetate-1-C^{14} by L-strain mouse fibroblasts in suspension with and without serum was also reported by King *et al.* (1959). These authors noted, however, that, in the presence of serum, large amounts of lipids were transported into the cells from the extracellular fluids.

After addition of Metrazol to the medium, incorporation of acetate into cholesterol was reduced. The sterol level in the presence of Metrazol was 56% below the level of sterols in control cultures. Despite this low level of cholesterol, the cells continued to thrive and to multiply. Cells grown in the presence of Metrazol and incubated in a Metrazol-free medium after "washout" of the drug rapidly synthesized sterols from acetate, indicating that Metrazol inhibited sterol biogenesis. If Metrazol acted only by enhancing the rate of degradation or excretion of cholesterol without affecting the rate of synthesis, removal of the drug from the environment would be expected to reduce the enhancement. Removal of the drug had an effect greater than mere restoration of the original metabolism. It stimulated the rate of synthesis in an attempt by the cell rapidly to restore the normal sterol level.

Several hypotheses may be offered to explain the Metrazol effect. The drug may inhibit a specific reaction on the pathway from acetate to cholesterol. It may limit the amount of oxygen available to the cell. It may produce a change in the cell wall which permits increased excretion of cholesterol into the medium. It may even increase uptake of cholesterol from the medium and cause inhibition of endogeneous synthesis through a form of feedback mechanism. The existence of such a feedback mechanism has been demonstrated recently by Bailey *et al.* (1961).

Many inhibitors of cholesterol synthesis have been reported. Some, such as α-*p*-biphenylbutyric acid, inhibit synthesis from acetate or mevalonate by competing for CoA or other essential co-factors (Tavormina and Gibbs, 1957). Others, such as triparanol (MER 29), do not inhibit utilization of acetate in sterol synthesis but interfere with reduction of desmosterol (Avigan *et al.*, 1960). Desmosterol does not precipitate with bromine and presumably it is a component of the "high counting companions" of cholesterol described by Schwenk *et al.* (1955). The purification procedure in our own experiments indicated that no significant amounts of "high counting companions" accumulated under the influence of Metrazol. A specific inhibition by Metrazol, if any, must have taken place at an earlier stage.

Bensch *et al.* (1961) have shown that there is an inverse relationship between protein synthesis and cholesterol synthesis. A decrease in the former stimulated both synthesis and accumulation of cholesterol. Since we have shown that addition of Metrazol stimulates cell division, it is not surprising to find that it caused a decrease in the rate of synthesis and accumulation of lipids within the cell. Our cultures used less oxygen per cell in the presence of Metrazol than did control cultures. While the over-all process of converting 15 molecules of acetate to one of cholesterol does not require additional oxygen, individual steps in the process depend on an adequate oxygen supply. The cyclization of squalene and the removal of the three "extra" methyl groups are among the processes which require oxygen (Cornforth, 1959). This may account for the decrease in oxygen utilization accompanying inhibition of sterol synthesis.

Warburg (1956) has postulated that when mammalian cells, which obtain most of their energy through respiration, are forced to rely on the much less efficient fermentation as the main source of energy, they compensate by an increase in the rate of growth. It is possible that Metrazol inhibits respiration and thereby speeds up cell growth. If this is true, metabolism of our fibroblasts under the influence of Metrazol should resemble the metabolism of cells derived from strongly carcinogenic tissue. This should be reflected in the pattern of cholesterol synthesis, which should be lower in tumors than in comparable normal tissues. Synthesis of cholesterol in tumors has been discussed extensively in the literature (Haven and Bloor, 1956; Schwenk and Stevens, 1958). It is difficult to ascertain whether tumor tissue synthesizes cholesterol because tumors in intact animals trap metabolites from the circulation. Presence of radioactive cholesterol in tumor tissue after an injection of radioactive precursors, therefore, is not proof of synthesis *in situ*. Since it is difficult to obtain tumors free from adherent normal tissue, most *in vitro* studies are equally inconclusive. Schwenk and Stevens (1958) incubated Yoshida ascites tumor cells with acetate-1-C^{14} and found that these cells did not synthesize more than traces of cholesterol. King *et al.* (1959) found that the cholesterol content of ascites cells increased 200–300% upon addition of *p*-fluorophenylalanine, an inhibitor of protein synthesis. Thus, rapidly growing mammalian cells, whether tumor cells or non-tumor cells, influenced by a metabolic stimulant such as Metrazol, apparently do not synthesize cholesterol, although they may be capable of such synthesis once rapid growth is interrupted. A survey of cholesterol synthesis in cultures of cell lines derived from normal and tumor tissues is under way in our laboratory.

The pattern of sterol biogenesis in our mammalian cells was generally similar to the pattern of sterol synthesis in yeast. Yeast cells grown in the absence of air contained only 10% of the sterols present in cells grown with aeration. When anaerobic yeast was aerated, sterols were rapidly produced until the level of aerobic yeast was reached. This property has been utilized in synthesizing highly radioactive yeast sterols (Klein *et al.*, 1954).

We have shown that the sterol pattern of aerobically grown yeast in the presence of Metrazol resembled that of anaerobically grown cultures. The sterol level was reduced, whereas squalene content was elevated. Washing out the drug produced the same effect as forced aeration of anaerobic cultures. Rapid synthesis of sterols ensued until the sterols reached the level characteristic of control aerobic cultures (Alexander and Alexander, 1961b). Mammalian cells in tissue culture, unlike yeast, could not be grown anaerobically, but addition of Metrazol had the same general effect. Cells grown in the presence of the drug utilized less oxygen per cell. Like anaerobic yeast, they had a low sterol content but were capable of proliferating freely. Removal of the drug resulted in rapid synthesis of sterols until the level normally present in cells grown in Metrazol-free media was reached.

Although these results do not show the specific site of Metrazol action, they are consistent with the hypothesis that Metrazol impairs the ability of the cells to utilize oxygen. Whether this is the mechanism by which Metrazol acts *in vivo* remains to be determined.

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Identification of Vitamin $K_{2(35)}$, an Apparent Cofactor of a Steroidal 1-Dehydrogenase of *Bacillus sphaericus**

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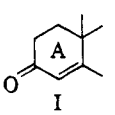
The cells of *Bacillus sphaericus* (MB431) were subjected to alkaline saponification in the presence of pyrogallol. Solvent extraction yielded an orange-colored residue which was subjected to two chromatographic purifications on silica gel and final chromatography over magnesium-aluminosilicate (Decalso). Recrystallizations of appropriate eluates yielded a yellow crystalline product compound which was identified as vitamin $K_{2(35)}$. The identification was based upon analytical and spectral evidence, including the nuclear magnetic resonance spectrum and comparison with synthetic members of the vitamin K_2 group. No evidence for the presence of a member of the coenzyme Q group was observed. Vitamin $K_{2(35)}$ has been tested for coenzymatic activity in the microbial 1-dehydrogenation of a Δ^4-3-ketosteroid; activity was observed. Menadione, hexahydrocoenzyme Q_4, and 6-phytyl-2,3,5-trimethyl-1,4-benzoquinone were also active. Corresponding coenzymatic activity of coenzyme Q_{10} in steroidal dehydrogenation in mammalian tissue, such as adrenal, may now be considered.

The therapeutic advent of 1-dehydrocortisone and 1-dehydrocortisol was soon followed by the microbial introduction of the Δ^1-double bond into Δ^4-3-ketosteroids by Vischer *et al.* (1955b) using *Didymella lycopersica*, Vischer *et al.* (1955a) using *Calonectria decora*, and Nobile *et al.* (1955) using *Corynebacterium simplex*. In addition, Lindner *et al.* (1956) using *Bacterium subtilis* and Sutter *et al.* (1957) using either *Mycobacterium laticola* or *Septomyxa affinis* accomplished the same type of steroidal transformation.

Stoudt *et al.* (1955), of this laboratory, reported on the selective microbial 1-dehydrogenation with a bacterial culture belonging to the species *Bacillus sphaericus*. In fact, these investigators successfully used ten steroidal substrates (I) and obtained the corresponding $\Delta^{1,4}$ dienone analogues (II) with this organism. The steroids were cortisone, cortisol, corticosterone, deoxycorticosterone, progesterone, 4 - androstadiene - 3,17-dione, and related compounds.



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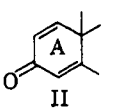

II

Using cell-free extracts from *Bacillus sphaericus*, Hayano *et al.* (1961) reported studies on the mechanism of the reaction and on the nature of the coenzyme involved with the steroidal 1-dehydrogenase. Although menadione was active in catalytic quantities, it was believed that the natural cofactor is probably a quinone of similar structure to menadione. Quinones of the vitamin K and coenzyme Q groups were of interest for evaluation.

We have undertaken an examination of the presence or absence of quinones of the vitamin K and coenzyme Q groups in cells of *Bacillus*

* Coenzyme Q. XXXV.