THE ASSIMILATION OF CHOLESTEROL BY MYCOBACTERIUM SMEGMATIS[1]

H. SOBEL AND A. PLAUT

Pathological Laboratory, Beth Israel Hospital, New York, N. Y.

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An investigation of the possible role of *Mycobacterium smegmatis* in the genesis of penile cancer followed the observation that horse smegma is carcinogenic (Plaut and Kohn-Speyer, 1947). It was intended to determine whether extracts of the organism possess carcinogenic activity[2] and whether the organism could transform cholesterol into a carcinogenic substance. During a study of the latter aspect of the problem, evidence for unsaturation of cholesterol by the organism was sought since this is part of a theoretical mechanism for the conversion of sterols into carcinogens in biological materials (Cook, 1933).

It is well established that a number of bacterial organisms can metabolize steroids, whether this action be limited to the oxidation of hydroxyl groups or entails a breakdown of the nucleus. Among these organisms it has been reported that mycobacteria can attack cholesterol. Tak (1942) found that mycobacteria in soil are able to decompose cholesterol and that other mycobacteria may grow in media containing cholesterol as its sole source of carbon. Turfitt (1947) has confirmed this and has observed that *M. smegmatis* is similar to *Mycobacterium phlei* in that some strains are fairly vigorous in this respect but that this property is entirely lacking in the majority and cannot, furthermore, be stimulated by enrichment cultures. It was found that the strain of *M. smegmatis* used in this report consumed cholesterol quite avidly. This is of added interest in that it concerns an organism commonly associated with human physiology.

EXPERIMENTAL RESULTS

Method. The finely powdered steroid was suspended in medium I of Turfitt (1944). In some cases it was first dissolved in methanol, which was then blown off under a stream of nitrogen. This modification offered no advantage. Dehydrated heart infusion broth (Difco) was added to a concentration of approximately 333 mg per liter of medium. The container was autoclaved and inoculated with a culture of *M. smegmatis*, strain 178 (American Type Culture Collection). It was incubated at 37.5 C. After completion of the experiment, the viability was tested by subculture on 1 per cent glycerol agar and observed for 6 days. The contents of the flask were extracted with ether and the ether-soluble residue was weighed.

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[2] Twenty-five mice are being treated with the residue of an alcoholic extract of *M. smegmatis* cultures. After 40 weeks one mouse had a carcinoma, after 47 weeks another mouse a papilloma.

Action on steroids. The organism has little ability to decompose cholesterol in the absence of supplements, but in the presence of heart infusion broth it does so quite vigorously (table 1). Glycine and cystine may be substituted for the heart infusion broth, but decomposition then is less vigorous. This may indicate a requirement for a specific nutrient for optimal growth or optimal cholesterol metabolism.

TABLE 1

Action of M. smegmatis on steroids

SUBSTANCE	QUANTITY	SUPPLEMENT TO INORGANIC BUFFER	TIME OF INCUBATION	VIABILITY AT COMPLETION OF EXPERIMENT	APPROXIMATE AMT. OF MATERIAL CONSUMED*
	mg		*months*		*mg*
Cholesterol	200	Heart infusion†	3	+	92
	200	Heart infusion	4	+	115
	400	Heart infusion	3	+	143
	400	Heart infusion	3	+	312
	3,000	Heart infusion	3	+	1,011
	200	Cystine	3	+	44
	200	Cystine	3	+	48
	200	Glycine	3	+	64
	200	Glycine	3	+	68
Chlorocholesterol	200	Heart infusion	3	+	0
	200	Heart infusion	3	+	1
Progesterone	100	Heart infusion	3	+	3
	100	Heart infusion	3	+	3
Dehydroisoandrosterone	150	Heart infusion	4¾‡	−	0
	150	Heart infusion	4¾‡	−	0

* Representing the difference between the quantity used and the amount of lipid-soluble residue recovered.

† In the absence of added supplement no growth was obtained and none of the steroid was consumed.

‡ After 2 months there was no evidence of growth. The flasks were reinoculated at this time; there was slow growth after several days but no viability at the end of the period.

Growth is vigorous in the presence of chlorocholesterol although this substance is not attacked. This is also true of progesterone. On the other hand, dehydroisoandrosterone appears to be inhibitory. This substance differs from cholesterol by the absence of the side chain, which is replaced by an oxygen atom. Cholic acid and desoxycholic acid are inhibitory, but these are not comparable substances since they are soluble under the conditions employed.

Although only a few substances were used, some interesting conclusions may be drawn. Members of the *Flavobacterium* group can oxidize nuclear hydroxyl groups of steroid compounds only if they are devoid of the cholesterol side chain (Molina and Ercoli, 1944). Turfitt (1944) has observed that organisms of the

genus *Proactinomyces* can decompose steroids having or lacking a side chain. It appears that *M. smegmatis* requires the presence of the side chain for action upon the steroid molecule, thus differing from the others. Furthermore, since the organism grows well in the presence of chlorocholesterol, which would be expected to be toxic if taken into the cell, and since dehydroisoandrosterone is inhibitory, it is possible that interaction takes place in two phases: that of penetration into the cell, and that of oxidative degradation following penetration. Each phase would be associated with different structural requirements. If this view is correct, dehydroisoandrosterone has the structural requirements necessary for penetration, but having entered the organism it interferes with certain physiological mechanisms. Since cholesterol is consumed, it must have both of the structural requirements.

The study of the action of microorganisms on steroids differs from most similar metabolic studies in that the substrate is insoluble in the medium. Indeed, the ability of the organisms to make the steroid available for action by its enzymes may be the conditioning factor in determining whether a given steroid is degraded, subjected to oxidation of its nuclear hydroxyl groups, or not attacked at all. This critical interaction probably occurs at the outermost layer of the cell, as a surface-film-monolayer reaction. Consequently the nature of the cell surface would determine the outcome of the over-all picture, and, therefore, different genera of bacteria might behave differently.

Action on cholesterol succinate. The interaction involving an insoluble substance is a very slow process. In view of this, a soluble form of cholesterol, that of the half-cholesterol ester of succinic acid, was used as substrate. The ester was suspended in water and dissolved with a minimal quantity of sodium carbonate solution. The buffer medium was added so that the final solution contained 5 to 10 mg of cholesterol in 3 ml. The organisms were obtained by washing with saline a 10-day culture of *M. smegmatis* on agar (heart infusion broth) into a "digestion tube." The saline was discarded and 6 ml of buffer medium were added. Then, 3 ml of cholesterol succinate solution were added. The pH of the solution was approximately 8. The tubes were shaken at 38 C for 24 hours. The reaction was stopped by adding 5 g of potassium hydroxide, and the contents of the tube were saponified by heating on a steam bath for 3 hours. The cholesterol was extracted and determined colorimetrically. The results are shown in table 2.

Cholesterol is consumed with great rapidity when in solution. Because of the relatively short time required, this technique should be suitable for the study of the intermediary metabolism of this substance. Acetone-dried preparations still retain some activity. It would be interesting to learn whether organisms that were unable to attack cholesterol in the solid phase can do so when it is in a solution.

Investigation of the unconsumed residue. In order to investigate the products of reaction and to determine whether unsaturation of the cholesterol nucleus could be demonstrated, the ether-soluble residue from a 3,000-mg batch (table 1) was fractionated. The bacterial residue after saponification yielded material

equivalent to 3 mg of cholesterol as determined colorimetrically. An equal quantity of organisms not exposed to cholesterol contained only a trace.

The ether-soluble residue was extracted with alkali and then treated with succinic anhydride in pyridine, according to the usual procedure, to obtain alcoholic and nonalcoholic fractions. These fractions were in turn treated with the Girard reagent to separate the ketonic material. The yields obtained with the respective fractions were as follows: alcoholic nonketonic 1,667 mg; alcoholic ketonic 2 mg; nonalcoholic ketonic 17 mg; nonalcoholic, nonketonic 26 mg; and alkali-soluble 34 mg.

The alcoholic nonketonic fraction was found to contain only cholesterol. No evidence was found to suggest that any substance was present that possessed more than one double bond per molecule. The alcoholic ketonic fraction and

TABLE 2

Action of M. smegmatis on cholesterol succinate

EXPT.	VIABILITY OF ORGANISMS	CHOLESTEROL SUCCINATE	CHOLESTEROL RECOVERED	CHOLESTEROL CONSUMED
		mg	*mg*	*mg*
1	+*	7.75	6.45	1.30
2	+	8.55	6.05	2.50
3	+	8.55	6.60	1.95
4	+	6.70	4.45	2.25
5	+	6.85	4.95	1.90
6	−†	6.70	6.20	0.50
7	−†	6.85	6.05	0.80
8	+‡	6.60	6.60	0
9	+	0	Trace	

* Organisms obtained from slants; all others from Kolle flasks.

† Acetone-dried powder.

‡ Treated immediately with KOH.

the ketonic nonalcoholic fraction were uncrystallizable gums. In the latter a small quantity of cholestenone-3 was demonstrated. The nonketonic, nonalcoholic fraction was subjected to chromatographic separation and a hydrocarbon was obtained that after crystallization from methyl alcohol yielded prisms melting at 119 to 120 C. It gave a positive Liebermann-Burchard reaction. It possessed no characteristic absorption maxima in its ultraviolet spectrum.

Turfitt (1948) has been able to isolate 3-ketoetiocholenic acid and 5-keto-nor-(−4)-4-coprostanic acid-3 from the reaction of *Proactinomyces erythropolis* on cholestenone using a rather ingenious technique that allows for continuous removal of breakdown products. Similar breakdown products were sought in the alkali-soluble fraction. It was separated into two fractions; one, an uncrystallizable gum that yielded soluble potassium salts, and the other, crystalline material the potassium salts of which were insoluble in water. The latter was separated from a minute amount of ketonic gum, which gave a positive Liebermann-Burchard reaction, and from a nonalcoholic oil that yielded a yellow color with the same reagent. The alcoholic substance was completely precipitable

with digitonin, indicating that ring A was intact. It yielded prisms melting at 135 to 137 C and gave a positive Liebermann-Burchard reaction.

It is very tempting to accept these acid derivatives of cholesterol as intermediary breakdown products in its metabolism by *M. smegmatis*. Although this may be true, such conclusions are open to uncertainty for the following reason: Cholesterol is generally considered to be a very stable substance, but it is well established that cholesterol in colloidal aqueous solution is very susceptible to the action of molecular oxygen (Bergstrom and Wintersteiner, 1941). Although these oxidations have been demonstrated to occur upon the intact steroid nucleus, a search for nuclear breakdown products appears not to have been made.

To check this point the following experiment was performed: Three 1,000-mg samples of cholesterol dissolved in a minimal amount of hot alcohol were poured into 200 ml of buffer medium in large flasks, and several drops of sorbitan monooleate were added to each flask. The mixtures were oxygenated for 24 hours. The product was taken up in ether and the acids were obtained by extraction with alkali. The acid fraction weighed about 25 mg and undoubtedly contained some acids from the sorbitan monooleate. The acids yielding insoluble potassium salts were isolated. Although crystallization could not be effected, it was found that this material gave a positive Liebermann-Burchard reaction.[3] Even this simple experiment must arouse caution in interpreting the presence of the above-mentioned acid breakdown products as a direct result of bacterial metabolism. Since a study of the action of microorganisms on cholesterol involves prolonged exposure to air and sometimes to oxygen, such experiments must be carefully controlled.

Action on procarcinogens. In pursuance of the idea that *M. smegmatis* might be involved in the genesis of penile cancer similar experiments were set up replacing the steroids with the following hydrocarbons: phenanthrene, methylcholanthrene, hexahydromethylcholanthrene, dehydronorcholene, and squalene. Hexahydromethylcholanthrene is a reduction product of methylcholanthrene (Wieland and Dane, 1933). Dehydronorcholene is an intermediate in the production of methylcholanthrene from desoxycholic acid (Wieland and Schlichting, 1925). Squalene and its cyclization product(s) have been found to be present in horse smegma. It has been postulated[4] that aromatization of the latter substance(s) could yield methylated chrysenes that are carcinogenic.

Evidence for conversion was sought by a study of the fluorescent spectra. Although growth of *M. smegmatis* was vigorous in the presence of phenanthrene and methylcholanthrene, no change in the fluorescent spectra was visible after 3 months. The fluorescent spectrum of hexahydromethylcholanthrene is similar to that of methylcholanthrene but shifted to the red. If the latter substance had been formed, it would have been easily detectable. Although growth was vigorous, methylcholanthrene was not found after 10 weeks. Dehydronorcholene remained unfluorescent after 3 months' exposure to the organism, although growth was vigorous in its presence. Squalene proved to be inhibitory, probably because of the formation of peroxides, which occurs readily with this

[3] Sorbitan monooleate does not give a positive reaction.

[4] In preparation.

substance. Both the control and the experimental material contained the fluorescent substance that forms in squalene on standing.

Action of Mycobacterium tuberculosis. A few preliminary experiments indicate that *M. tuberculosis* is capable of consuming cholesterol, but much less vigorously than *M. smegmatis.* Possibly this is related to its slower growth. *M. tuberculosis* was grown in the presence of 10-mg samples of cholesterol in Dubos medium and on buffer medium with heart infusion broth for 3 months. Approximately 28 per cent had been consumed in the former medium and 22 per cent in the latter. With 200-mg samples of cholesterol in medium containing heart infusion broth, 3 mg disappeared after 3 months and 14 mg after 6 months. It remains to be seen what role this bacterial breakdown of cholesterol plays in pathological processes.

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SUMMARY AND CONCLUSIONS

Mycobacterium smegmatis has been observed to consume cholesterol actively when this substance is in the solid phase or in solution as its succinate. The presence of the side chain appears to be necessary for this action, thus differentiating this organism from several others in respect to its action on steroids. An attempt to find evidence for the dehydrogenation of the steroid nucleus was unsuccessful, as were attempts to demonstrate the conversion of several other theoretical procarcinogens into carcinogens. Several unidentified breakdown products of cholesterol were found, and it is pointed out that acid derivatives might be artifacts produced by molecular oxidation rather than bacterial metabolites.

REFERENCES

BERGSTROM, S., AND WINTERSTEINER, O. 1941 Autoxidation of sterols and colloidal aqueous solution. J. Biol. Chem., **141**, 597–610.

COOK, J. W. 1933 Discussion on tumours. Proc. Roy. Soc. (London), B, **113**, 273–277.

MOLINA, L., AND ERCOLI, A. 1944 Sui batteri che determinano l'ossidazione dei steroidi ad attività ormonale. II. Isolamento e caratteristiche di un nuovo stipite di flavobacterium: il flavobacterium carbonilicum. Boll. ist. sieroterap. milan., **23**, 164–167.

PLAUT, A., AND KOHN-SPEYER, A. C. 1947 The carcinogenic action of smegma. Science, **105**, 391–392.

TAK, J. D. 1942 On bacteria decomposing cholesterol. Antonie Leeuwenhoek J. Microbiol. Serol., **8**, 32–40.

TURFITT, G. E. 1944 The microbiological degradation of steroids. 2. Oxidation of cholesterol by *Proactinomyces.* spp. Biochem. J., **38**, 492–496.

TURFITT, G. E. 1947 Microbiological agencies in the degradation of steroids. II. Steroid utilization by microflora of soils. J. Bact., **54**, 557–562.

TURFITT, G. E. 1948 The microbiological degradation of steroids. 4. Fission of the steroid molecule. Biochem. J., **42**, 376–383.

WIELAND, H., AND DANE, E. 1933 Untersuchungen über die Konstitution der Gallensäuren. LII. Über die Haftstelle der Seitenkette. Z. Phys. Chem., **219**, 240–244.

WIELAND, H., AND SCHLICHTING, O. 1925 Untersuchungen über die Gallensäuren. XXIV. Über den Vorgang der Wasserabspaltung unter Bildung neutraler Substanzen. Z. Phys. Chem., **150**, 267–275.