morphological, optical, and x-ray crystallography.

Study of structure determinations of diopside (*11*) and tremolite (*12*) and packing models of both shows that the amphibole space group directly comparable to diopside $C2/c$ is $I2/m$. Lamellae directly comparable to (001) in pyroxene would be indexed as $(\bar{1}01)$ for space group $C2/m$ of amphibole. Similarly, if the C cell is chosen for the amphibole, then the directly corresponding pyroxene space group is $I2/c$. Figure 2 shows the relations between these two alternative sets of pyroxene and amphibole cells together with the Z-vibration directions and exsolution planes. The traditional optic orientation of diopside was independently confirmed on a chromian specimen from Outokumpu, Finland, showing excellent {001} parting. The angle $Z \wedge c$ has the same sense relative to the internal structure in both diopside and clinoamphibole.

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Sterol Requirement for Reproduction of a Free-Living Nematode

Abstract. *The free-living, hermaphroditic nematode* Caenorhabditis briggsae *has a nutritional requirement for sterols. It will reproduce indefinitely in a liquid medium containing only bacterial cells* (Escherichia coli) *and salts if various sterols are present. Several other lipid-soluble materials are ineffective in supporting reproduction.*

Reproduction of free-living nematodes under axenic conditions requires the presence of an undefined tissue extract (*1*). Mammalian liver or chick embryo are the most appropriate sources (*2*), though extracts of other tissues and bacteria are reportedly effective (*3*). Although a suitable defined basal medium for the organisms has been developed, the nutritional nature of the extract has not been satisfactorily resolved.

Fractionation of an extract of lamb liver on hydroxylapatite has led to the recovery of fractions which support growth and reproduction of free-living nematodes (*3*). These fractions consist mostly of protein, but they also contain a small lipid moiety. Since the basal medium contains no lipids (*4*), the nematodes can either synthesize all of their required sterols and fatty acids or these materials must be provided by the "growth factor." The free-living nematode *Turbatrix aceti* can synthesize polyunsaturated fatty acids *de novo* (*5*), but no evidence was found for sterol biosynthesis in this organism or in *C. briggsae* (*6*).

Caenorhabditis briggsae, a hermaphroditic nematode, grows and reproduces in the presence of *Escherichia coli* (and other bacteria) on a nutrient agar medium. However, Dougherty (*7*) reported that the nematode will not reproduce if the nutrient agar is changed to one containing only basal salts and glucose. These results suggest that essential metabolites lacking in the bacteria are provided in the nutrient agar. Particularly significant is the fact that procaryotic bacteria are deficient in sterols and polyunsaturated fatty acids (*8*).

Axenic larvae of *C. briggsae* were inoculated into sterile, capped test tubes (13 by 100 mm) containing slants of minimal agar previously streaked with *E. coli* (ML30 strain) grown on a minimal medium consisting of salts and glucose. A purified grade of agar (Difco Purified Agar) was used and contained the following: 8.5 g of K_2HPO_4, 4.2 g of KH_2PO_4, 1.0 g of NaCl, 0.8 g of NH_4Cl, 0.1 g of Na_2SO_4, 0.05 g of $MgCl_2 \cdot 6H_2O$, 0.01 g of $CaCl_2$, 0.0005 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of glucose, and 16 g of agar per liter of distilled water, adjusted to *p*H 6.9 to 7.0. The nematodes grew and reproduced in 5 days at 20°C. The offspring were serially subcultured four times without diminishment of growth. Thus, it appeared that our agar possessed an essential nutrient lacking in Dougherty's original preparation (*7*), or that the nematodes could grow successfully on *E. coli* as the sole source of organic nutrients. However, larvae of *C. briggsae*, when inoculated into a liquid medium limited to phosphate buffer and *E. coli* (see below) reproduced readily for only one generation. The offspring usually grow only to advanced larval stages or to the size of small adults. An occasional individual (about one out of six) managed to produce new larvae which did not survive further subculture in this medium.

To show that the material lacking in the liquid medium is a sterol, we carried out the following experiments. *Escherichia coli* (ML30) was grown in a minimum liquid medium with the same composition as that of the agar slants (except that 2.5 g of glucose per liter was added) for 9 hours at 37°C. The cells were collected, washed by centrifugation, and finally diluted with a .067*M* potassium phosphate buffer–salt solution containing in final concentration: 3.25 m*M* NaCl, 3.0 m*M* magnesium citrate, 1.5 m*M* $CaCl_2$, 0.235 m*M* Na_2SO_4, 0.15 m*M* $FeCl_3$, 0.112 m*M* $MnCl_2$, 0.075 m*M* $ZnCl_2$, and 0.038 m*M* $CuCl_2$. The concentration of bacterial cells was approximately 0.3 mg/ml (dry weight).

Stoppered tubes (10 by 75 mm) containing 0.25 ml of cell suspension were each inoculated with three freshly hatched larvae and incubated at 20°C. The offspring from these cultures were subcultured into fresh medium of the same composition. From the occasional individual which again reproduced, "depleted" larvae were obtained for the growth experiments outlined in Fig. 1. Cholesterol, 7-dehydrocholesterol, ergosterol, β-sitosterol, and stigmasterol were combined and dissolved in Tween 80, and the product was sterilized by Millipore filtration. The

combined sterols were added to the bacterial medium so that each sterol was present at a concentration of 10 μg/ml (50 μg/ml total); Tween 80 comprised 0.13 percent.

The larvae inoculated into the medium containing *E. coli* cells plus the sterol mixture reproduced rapidly and in large numbers (usually 70 to 100 larvae in 3 days), whereas those transferred into medium containing cells alone or with Tween did not (Fig. 1). Furthermore, after "depleted" larvae exposed to sterols reproduce, the offspring are capable of producing at least one generation in the absence of these compounds.

The medium containing both bacteria and sterols can sustain indefinite subculture: thus far, *C. briggsae* has been grown through ten successive subcultures, with consistently rapid and profuse growth. We often observed generation times of less than 2.5 days, a rate which equals or exceeds the best so far reported for *C. briggsae* (*9*).

Similar nutritional experiments were carried out with the individual sterols. Cholesterol, ergosterol, 7-dehydrocholesterol, β-sitosterol, and stigmasterol were tested at a concentration of 25 μg/ml in the presence of *E. coli*. Cholestane was also tried. In each instance, reproduction was followed through at least three successive subcultures of larval offspring. In the control experiments (cells alone) only an occasional individual reproduced, and the offspring, after subculture, died. Those media containing ergosterol, 7-dehydrocholesterol, β-sitosterol, and stigmasterol supported rapid reproduction, and populations were achieved comparable to those obtained with the sterol mixture. Addition of cholesterol led to the production of smaller populations. Cholestane was almost as effective as the other sterols in achieving reproduction, but the larvae did not produce the usual single generation when subcultured into a medium containing cells alone. This may indicate that, unlike the sterols, cholestane cannot be carried over in adequate amounts for one generation.

Several other lipid-soluble materials were tested for their ability to support reproduction. Squalene alone, vitamin A alone, and a mixture of vitamin E, linoleic acid, and arachadonic acid failed to support reproduction, being no better than the control experiments.

We conclude that the free-living nematode *C. briggsae* requires a sterol or related derivative for reproduction. It is not clear, however, which sterol best fills this requirement. Necessary quantities appear to be small, as good growth and reproduction are achieved at a concentration of 1.3 μg of the sterol mixture per milliliter. At lower concentrations, reproduction was irregular. Bacterial cells are obviously present in excess, since the tubes that contain adequate amounts of sterols eventually develop populations in the hundreds.

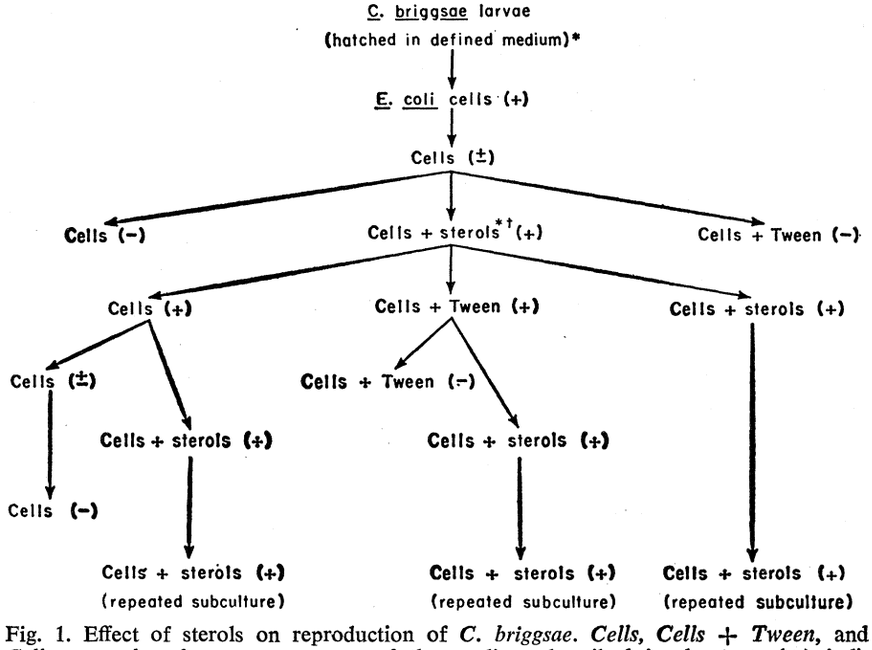

Fig. 1. Effect of sterols on reproduction of *C. briggsae*. *Cells*, *Cells + Tween*, and *Cells + sterols* refer to components of the medium described in the text; (+) indicates rapid reproduction (2.5 to 3 days) by all individuals and the production of many offspring (70 to 100) from the original three larvae; (±) indicates that an occasional nematode (about one in six) yielded a second generation when grown in medium containing cells alone; (−) indicates the absence of reproduction. In each experiment duplicate tubes were observed, each initially containing three larvae. * Adult nematodes were rinsed in several changes of buffer solution, and the freshly hatched larvae then used in the subsequent experiments. † Sterols were dissolved in Tween 80.

Since *C. briggsae* obtains all of its nutritional requirements except sterols when grown on *E. coli*, they apparently do not require polyunsaturated fatty acids. This result is in agreement with the report that the free-living nematode *T. aceti* can synthesize these products *de novo* (*5*).

Our data are subject to two criticisms: (i) It is possible, though unlikely, that the bacteria convert the sterols in the medium to other derivatives essential for the nematodes. A final solution to this problem awaits a cell-free preparation from bacteria which will replace living cells as food for *C. briggsae*. (ii) Impurities in the sterol preparations might be responsible for the growth activity; gas-liquid chromatography of the sterols showed the presence of significant amounts of impurities in all except cholesterol and cholestane. However, the likelihood of a specific impurity in each preparation seems limited since the sterols were derived from widely different sources (animal, plant, and yeast).

The establishment of a sterol requirement for *C. briggsae* has relevance to the problem of identifying the unique growth supplement from liver necessary for the axenic culture of this and other free-living nematodes. One can conclude that the "active" liver fractions isolated by Sayre (*3*) consist of at least two essential components, one of which is a sterol (*10*). The other constituent is presumably the heat-labile substance which also exists in bacteria and chick-embryo extract (*2*). Implicit in this conclusion is the fact that the protein fractions, previously found to be "inactive," may merely be lacking in a lipid (sterol-containing) moiety. The clear identification of a sterol requirement for *C. briggsae* should simplify future efforts to characterize the factor or factors which are necessary for the continuous reproduction of this and related organisms.

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Acetylation of Human Serum Albumin by Acetylsalicylic Acid

Abstract. *Human serum albumin is acetylated when exposed to acetylsalicylic acid (aspirin) under physiologic conditions in vitro. Indications are that a similar phenomenon occurs in vivo after ingestion of aspirin.*

Previous studies have shown that treatment of human serum albumin (HSA) with acetylsalicylic acid (aspirin) permanently enhanced the capacity of albumin to bind a marker anion, I^{131}-labeled acetrizoate (*1–4*). The treatment in vitro or in vivo of albumin by salicylic acid did not produce this effect. The permanent alteration of the capacity of albumin to bind the marker anion indicated that the structure of HSA was irreversibly altered by aspirin. We now report that aspirin acetylates HSA under physiologic conditions in vitro and that acetylation of HSA in vivo is probably responsible for the reported enhancement of acetrizoate binding observed in the serum of many patients (*1*) or normal subjects (*4*) ingesting aspirin in a therapeutic dosage.

The inherent chemical instability of the aspirin molecule, as exemplified by its spontaneous hydrolysis in water to acetic and salicylic acids, suggested that either the acetyl or the benzoate portion of aspirin might interact with HSA. Accordingly, HSA was treated with aspirin labeled with C^{14} either at the carboxyl group or at the acetyl group; sodium salicylate labeled with C^{14} at the carboxyl group was used as a control (*5*) (Fig. 1). Human serum albumin (Squibb, fraction V); 14 mg/ml, $2.0 \times 10^{-4}M$ was dialyzed against $0.10M$ sodium phosphate buffers, *p*H 5.0 through 10.0. Duplicate 2.0-ml samples of these albumin preparations were added to 2.0 ml of $1.0 \times 10^{-3}M$ solutions of aspirin labeled in the carboxyl or acetyl position, or of sodium salicylate labeled in the carboxyl position. The final reaction concentrations of the salicylates were chosen to be comparable to therapeutic blood levels. The mixtures were incubated at 37°C for 24 hours and then were dialyzed at 4°C for 48 hours against multiple changes of $0.15M$ NaCl containing $0.01M$ sodium salicylate as carrier to remove ionically bound aspirin. No radioactivity was detected in the final dialyzate. Duplicate 0.10-ml samples were counted for C^{14} activity in a Packard Tri-Carb liquid scintillation counter. Samples of 1.0 ml were dialyzed against three 1-liter volumes of $8M$ urea (*p*H 5.5) or $6M$ guanidine (*p*H 6.4) at 25°C for 48 hours, against $0.10M$ NaH_2PO_4 for 24 hours, and then against phosphate-buffered saline, *p*H 7.0, for 24 hours. The samples treated with urea and guanidine were counted for C^{14} activity. Protein concentrations were determined before and after treatment with urea or guanidine by the micro-Kjeldahl method of nitrogen determination. In addition to the above, duplicate 1.0-ml samples were dialyzed free of the sodium salicylate with borate-saline buffer, *p*H 7.4 and acetrizoate binding was determined by equilibrium dialysis with $1.0 \times 10^{-7}M$ I^{131}-labeled acetrizoate (*6*).

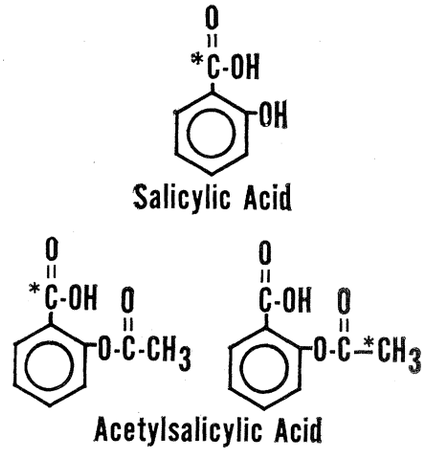

Fig. 1. Structural formulas of salicylic acid and acetylsalicylic acid showing position of carbon-14 label (*).

Human serum albumin treated with aspirin labeled in the carboxyl position retained a maximum of less than 2 moles of C^{14}-carboxyl per 100 moles of HSA (Table 1). It is possible that this extremely low binding could be due to the formation of a salicylamide derivative (*7*); alternatively the <1 percent trace contaminant that may have been in the aspirin labeled in the carboxyl position could account for this binding. The C^{14} activity of HSA treated with salicylic acid labeled in the carboxyl position was negligible. In striking contrast, the HSA treated with aspirin labeled in the acetyl position had significant C^{14} activity, indicating that acetylation of HSA occurred. The degree of acetylation was identical before and after treatment with either $8M$ urea or $6M$ guanidine and varied with the *p*H of the reaction mixture, tending to increase with decreasing hydrogen ion concentration. At *p*H 7.3 (that is, physiologic *p*H) an average of 1.2 acetyl groups were covalently bound per molecule of HSA, and a maximum binding of three acetyl groups per molecule was observed at *p*H 9 to 10. The degree of acetylation of HSA was determined by assuming that 100 percent of the C^{14} activity was associated with HSA rather than with a trace serum contaminant in the purified fraction V preparation. This assumption was verified by autoradiography of an immunoelectrophoretic pattern of HSA acetylated with acetylsalicylic acid-1-C^{14}(acetyl). Carbon-14 activity was associated with the entire albumin line and not with other serum proteins (Fig. 2), even when subjected to electrophoresis with whole normal human serum as carrier.

Binding of the marker anion, acetrizoate, was similarly enhanced by treatment with aspirin labeled in either the carboxyl or acetyl position. The acetrizoate data obtained with the latter is presented in Table 1. Near maximum binding was seen at *p*H 7.0, where there was an average of one acetyl group per HSA molecule. Despite the addition of two more acetyl groups at *p*H 9.0, there was a minimum increase in acetrizoate binding.

The striking difference in residual C^{14} activity which remained on the HSA molecule after exposure to aspirin labeled in the acetyl position as compared with aspirin labeled in the carboxyl position indicated that aspirin