pronounced change in the ratio of the *trans* to the *cis* forms, with the concentration of the former always being several times greater than that of the latter.

There was little evidence of double-bond migration under normal conditions. The conjugated linoleic acid and linolenic acid have retention volumes greater than those of the normal compounds and could be separated by gas-liquid chromatography (Daniels & Richmond, 1960). No conjugated linoleic acid and linolenic acid could be detected either during control periods or during periods in which oleic acid was infused.

Conjugates accounted for 5–10% of the polyenoic acids during infusion of small amounts of non-conjugated linoleic acid and linolenic acid and 15–25% during infusion of larger amounts. The conjugated polyenoic acids are hydrogenated with difficulty (Garton, 1959) and would be expected to accumulate to a much greater extent than the non-conjugated acids.

The above results offer no support for the hypothesis that the process of hydrogenation involves appreciable bond migration and accumulation of conjugated acids (Shorland *et al.* 1957; Kepler *et al.* 1965), but they are consistent with the

hypothesis that elaidic acid, and not oleic acid, is an intermediate in the hydrogenation of the polyenoic acids in the rumen.

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The Cytochemical Localization of Alkaline Phosphatase in *Escherichia coli* at the Electron-Microscope Level

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Malamy & Horecker (1961, 1964a,b) discovered that alkaline phosphatase (EC 3.1.3.1) of high specific activity is obtained in quantitative yield when *Escherichia coli* cells containing the enzyme are converted into spheroplasts by the action of EDTA and lysosyme. Their observations showed that the enzyme, which is synthesized after the release of an inorganic phosphate repressor mechanism, assumes a remarkably well-defined localization. In intact cells this enzyme behaves as though it were contained in a compartment separated from the external medium and not in complete equilibrium with it. Malamy & Horecker (1964a) suggested that the alkaline phosphatase lies in a compartment between the cell wall and

cell membrane. In this communication we report the demonstration by an electron-microscope technique that the enzyme is located in an intermediate layer of the cell wall, between the outer membrane and the plasma membrane.

Cells of *E. coli* strain W 3747, kindly supplied by Dr Frank Rothman, were cultured in the medium described by Levinthal, Signer & Fetherolf (1962). Here, de-repression occurs after an initial period of growth that is limited by the small amount of phosphate present. KH_2PO_4 (3.2 mm) was added to a 'control' culture to repress enzyme synthesis.

After 18 hr. the cultures were centrifuged at 8000g (10000 rev./min. in an International centrifuge). We used a modification of the method of

Sabatini, Bensch & Barnett (1963) for the cytochemical detection of phosphatase (Pollak & Shorey, 1964). The cells were fixed as a pellet for 1½ hr. at 5° in 6.5% (w/v) glutaraldehyde solution in 0.1M-sodium cacodylate adjusted to pH 7.4 with 0.1N-HCl. They were washed thoroughly with the sodium cacodylate buffer and stored in this buffer at 5° before incubation for 2 hr. at 37° with a substrate solution of the following composition: 25ml. of 2% (w/v) sodium β -glycerophosphate; 25ml. of 2% (w/v) sodium veronal; 5ml. of 2% (w/v) $\text{Ca}(\text{NO}_3)_2$; 5ml. of 0.8% (w/v) MgCl_2 ; 40ml. of 0.1M-sodium cacodylate buffer, pH 7.4.

After incubation the cells were treated with aq. 2% (w/v) $\text{Co}(\text{NO}_3)_2$. After this, the cells were post-fixed in 1% (w/v) OsO_4 for 2 hr. and then upgraded through a series of water-ethanol mixtures and embedded in Araldite. The Araldite blocks were sectioned on an LKB ultratome with glass knives and the sections were mounted on copper grids. Before examination the sections were stained with $\text{Pb}(\text{OH})_2$ by method A of Karnovsky (1961).

Controls were conducted by incubating the repressed cells in the substrate solution and also by incubating repressed and de-repressed cells in solution that contained no β -glycerophosphate but that was similar in other respects.

Electron-micrographs are shown in Plate 1 of cells with (a) and without (b) alkaline phosphatase. Electron-dense material is clearly visible in the first case, where it has been deposited between the outer membrane and the plasma membrane. No such electron-dense material was found in cells from the 'control' culture, and negative results were also obtained with both repressed and de-repressed cells when β -glycerophosphate was omitted from the incubation solution.

A study is being made of the effects of glutaraldehyde on this enzyme and preliminary results are relevant to the validity of the fixation technique. An enzyme preparation was made by the method of Garen & Levinthal (1960). Fractions from the DEAE-cellulose column that showed only the active isoenzyme protein bands after electrophoresis in starch gel were pooled. Small samples of enzyme solution were taken in a specially calibrated pipette, followed immediately by 5 vol. of glutaraldehyde

solutions in sodium cacodylate buffer. Such mixtures were added after various times to 500 vol. of *p*-nitrophenyl phosphate solution for enzyme assay (Garen & Levinthal, 1960). Control experiments showed that the addition of the glutaraldehyde solutions to substrate solution before the addition of enzyme had no measurable effect on the initial reaction rate.

The addition of glutaraldehyde has a biphasic effect on enzyme activity. In one experiment with a 6.5% solution, 39% of the activity remained after 5 min. and 31% after 30 min., but the activity measured as soon as possible after mixing was only 52% of the activity of the untreated enzyme solution. When the fixative solution was diluted eightfold, a 'zero-time' reading of 87% was obtained decreasing to 42% after 5 min. and 40% after 30 min.

Thus it seems possible that the glutaraldehyde rapidly changed the enzyme molecules into forms that retained detectable activity after fixation. Further evidence in support of this has been obtained from starch-gel electrophoresis of the enzyme preparation (Loke & Done, 1965) after treatment with glutaraldehyde. All of the protein fractions obtained had detectable enzyme activity, but with one exception their mobilities were different from those of the original isoenzymes. The most prominent fraction of the modified enzyme migrated much further than the untreated isoenzymes. This increased anodic mobility is consistent with the loss of amino groups after reaction with glutaraldehyde.

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Plate 1. Electron micrographs of *E. coli* strain W3747: (a) de-repressed cells; (b) repressed cells.
Magnification $\times 65000$.