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*INDUCTION OF CELLULAR DNA SYNTHESIS BY POLYOMA VIRUS**

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The transformation of normal cells into tumor cells by polyoma virus is caused by the interaction of susceptible cells with the DNA of the virus. Thus, purified polyoma virus DNA has been shown to transform cells cultured *in vitro*,[1] whereas the empty protein shells of the virus do not produce this effect.[2] Consequently, a knowledge of the functions of the viral genes is basic to an understanding of the mechanisms of cell transformation. With the hope of identifying these functions, we have initiated a study of the biochemical events which occur after the cytocidal infection of mouse kidney cells by polyoma virus. This article describes the effects of virus infection upon DNA synthesis and upon the activity of enzymes involved in DNA synthesis. One of the most interesting findings was that the virus induces the synthesis of *cellular* DNA in addition to viral DNA.

Materials and Methods.—Virus: The polyoma virus used was of the large plaque type.[3] It was grown and purified according to Winocour.[4] The virus was assayed by plaque formation on secondary mouse embryo cultures.

Cell cultures: The cells were derived from kidneys of 10–14-day-old mice. After trypsinization, a large number of cells (0.1 ml of packed cells per 100-mm Petri dish) were plated onto plastic Petri dishes in a reinforced[5] Eagle's medium with 10% horse serum. Within 24–48 hr after plating, the cultures consisted primarily of confluent layers of epithelial cells. These dense cultures had a very low DNA-synthesizing activity from the second day on.

Infection of cultures: The cultures were infected 48–64 hr after the plating of the cells. The medium was removed and 0.4 ml of a suspension of purified virus containing 5×10^9 PFU/ml was added. The cultures were incubated for 1 hr at 37°C in a well-humidified incubator flushed with a CO_2-air mixture; after this time fresh medium was added. All control cultures were mock-infected under identical conditions but without virus.

DNA synthesis: The amount of H^3-thymidine taken up into DNA was determined from the radioactivity in acid-insoluble material (H^3-thymidine did not label RNA). The amount of P^{32}-orthophosphate taken up into DNA was determined after SDS-phenol extraction of the cultures and alkali digestion of the RNA.

SDS-phenol extraction of DNA: Cell cultures were washed 4 times with Tris-buffered saline (TD:NaCl, 0.8%; KCl, 0.038%; Na_2HPO_4, 0.01%; Sigma 7–9, 0.3%); the cells were removed in a SDS-solution containing 0.15 *M* NaCl, 1.5×10^{-3} *M* Na-citrate, 0.1 *M* Tris buffer, pH 8.0, 0.5% sodium dodecyl sulfate (Coleman, Matheson, and Bell), and 5×10^{-3} Versene. After addition of Na-trichloroacetate to a final concentration of 0.3 *M*, the mixture was extracted 3

times with 80% phenol (in 20% 0.5 *M* Tris buffer, pH 8.0). The aqueous phase was extracted with 2 vol of ether and dialyzed against SSC/10 (SSC:0.15 *M* NaCl, 0.015 *M* Na-citrate).

Chromatography of DNA: The methylated albumin column was prepared according to the procedure of Mandell and Hershey.[6] Fractions were collected and precipitated with 5% trichloroacetic acid after the addition of 2 mg of bovine serum albumin.

The DNA-DNA hybridization: The DNA-DNA hybridization was carried out according to the procedure of McCarthy and Bolton,[7] except that final concentration of agar was 4%. The DNA for the agar column was isolated from baby mouse kidneys by SDS-phenol extraction; it was treated with RNAse, re-extracted, and collected by ethanol precipitation.

The concentration of DNA in the agar was 0.31 mg per gram of DNA-agar. Hybridization was carried out by incubating 0.3 mg of DNA-agar with 0.3 ml of heat-denatured DNA in 2 × SSC for 16 hr at 60°C. The DNA had been sonicated for 5 min in an MSE ultrasonic disintegrator. The mixture was then placed in a jacketed column and washed 10 times with 10-ml portions of 2 × SSC at 60°C in order to remove the unhybridized DNA. The hybridized DNA was subsequently eluted at 75°C with 5 washes of SSC/100 (10 ml each). To each fraction, 0.2 mg of herring sperm DNA and 1 ml of 100% trichloroacetic acid were added in the cold to precipitate the eluted DNA. The precipitates were collected on Millipore filters and counted in a liquid scintillation counter.

The *assay for infectious DNA* was carried out by plaque formation[8] after SDS-phenol extraction.

RNA synthesis: The amount of H^3-uridine taken up into cell RNA was determined by isolating the RNA mononucleotides after alkali digestion.

Protein synthesis: The amount of H^3-leucine taken up into acid-insoluble material was determined.

Enzyme assays: Thymidine kinase: Extracts were prepared by homogenizing cells in cold distilled water containing 2-mercaptoethanol (1/10,000 v/v); Tris buffer of pH 7.9 was added to a final concentration of 10^{-2} *M*, and the extracts were centrifuged for 10 min at 12,000 × *g*. The supernatants were assayed following the method of McAuslan[9] by using H^3-thymidine as substrate. The phosphorylated derivatives were separated from thymidine by paper chromatography in an isopropanol-NH_3 solvent.[10]

dCMP-deaminase: Extracts were prepared by homogenizing cells in a buffer solution containing 0.03 *M* phosphate buffer, pH 7.3, 2×10^{-4} *M*, $MgCl_2$, 3×10^{-2} *M* NaF, and 2×10^{-4} *M* dCTP. The assay conditions were similar to those of Maley and Maley[11] with H^3-dCMP as the substrate. dUMP was separated from dCMP by chromatography in a solvent consisting of 85% isopropanol, 15% 0.3 *N* HCl.

DNA polymerase: The extracts prepared for the thymidine kinase assays were used. The assay method was that of Keir[12] with heat-denatured mouse kidney DNA as primer; the labeled triphosphate was H^3-dATP.

Results.—Rate of DNA synthesis: DNA synthesis in mouse kidney cultures was studied either by following the incorporation of H^3-thymidine or P^{32}-orthophosphate, or by assaying for infectious DNA.

Table 1 shows the extent of incorporation of H^3-thymidine and of P^{32} into the DNA of infected and control cells between 16 and 30 hr after virus infection. The incorporation of either label was approximately 10 times higher in the infected cells than in the control cells. This result is in agreement with previous cytological findings.[13] The amount of DNA synthesized during this period in the infected cells was estimated from a comparison with the number of counts incorporated into growing mouse embryo cells under identical conditions of labeling within a period of doubling of total DNA. It was found to correspond to about 25 per cent of the pre-existing total cellular DNA, under the assumption that the efficiency of incorporation of H^3-thymidine into DNA was equal in the two cell systems.

The time course of incorporation of H^3-thymidine into the DNA of the infected cells and the appearance of infectious DNA are shown in Figure 1. In this experiment, the label was added immediately after virus adsorption. The results

show that the incorporation of H^3 into DNA is roughly the same in control and in infected cells for the first 16 hr. After this time period, however, there is a sharp increase in the rate of DNA synthesis in the infected cells. A rapid rate of DNA synthesis continues in these cells until about 40 hr after infection; at this time DNA synthesis appears to be turned off. Surprisingly, the formation of infectious DNA is not synchronous with the incorporation of H^3-thymidine into DNA.

TABLE 1

	P^{32} (cpm/culture)	H^3 (cpm/culture)
Infected culture	80×10^5	90×10^4
Uninfected culture	7.2×10^5	8×10^4

Incorporation of label into the DNA of infected and uninfected cells. Sixteen hr after virus absorption 0.25 mC of $Na_2HP^{32}O_4$ (neutralized, carrier-free) in 10 ml of phosphate-free medium was added to one infected and one uninfected culture. Similarly, 5×10^{-4} mmoles of thymidine and 0.25 mC H^3-thymidine (1.9 C/mmole) in 10 ml of medium were added to one infected and one uninfected culture. DNA was purified by SDS-phenol extraction. An aliquot was incubated with NaOH (1 *N*, 16 hr at room temperature) and precipitated with carrier bovine serum albumin in 5% trichloroacetic acid. Precipitate was collected and washed on a Millipore filter (grade HA) and counted in a liquid scintillation counter.

Nature of DNA synthesized: Viral and cellular DNA can be separated on a methylated albumin column presumably because the polyoma DNA is smaller than the extracted cellular DNA and uniform in size. Figure 2 shows the elution pattern of a mixture of P^{32}-labeled viral DNA isolated from purified virus and H^3-labeled cellular DNA from an uninfected culture of mouse kidney cells. The viral DNA elutes first from the column, relatively uncontaminated by cellular DNA.

In order to examine the nature of the DNA synthesized after viral infection, a

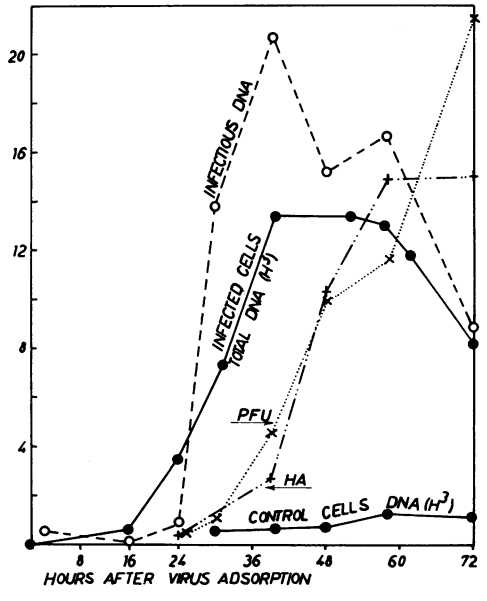

FIG. 1.—Time course of syntheses in the infected cells. Immediately after the adsorption period, 5 ml of medium containing 5×10^{-5} *M* thymidine and 25 mC of H^3-thymidine were added to each of several infected and uninfected cultures. At various times 2 infected and 2 control cultures were removed, and the amount of H^3 taken up into DNA was determined. Other cultures were infected and incubated similarly, but without adding H^3-thymidine. Pairs of these cultures were used for determining the titer of infectious DNA after SDS-phenol extraction. For determining the infectious titer (PFU) and hemagglutinating titer (HA) the cells were scraped off into the culture medium, which was then frozen-thawed three times. Every point represents the average of two determinations. The scale of the ordinate is linear, in arbitrary units. One unit corresponds to 6.7×10^2 PFU of infectious DNA, to 10^9 PFU of virus, and to 10^4 hemagglutinating units per culture.

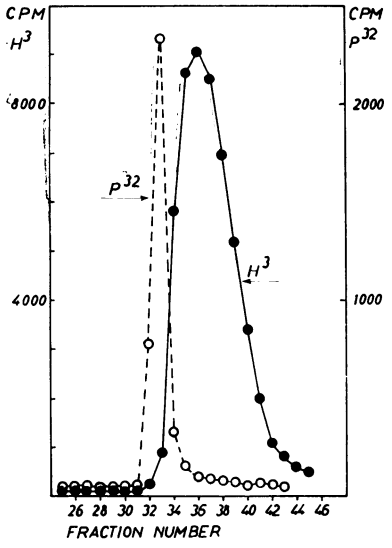

FIG. 2.—Fractions from a column of methylated albumin. The H^3-labeled infected cell DNA (● ——) is from the experiment described in the legend to Table 1. The P^{32}-labeled viral DNA (○ - - -) was phenol-extracted from a purified virus preparation. The total number of counts present in each fraction is given.

mixture consisting of DNA isolated from infected cells and DNA isolated from noninfected cells was chromatographed on a column of methylated albumin. The DNA from the infected cells was labeled by a pulse of H^3-thymidine from 16 to 30 hr after virus adsorption. The control cellular DNA was labeled with P^{32} by giving to an uninfected culture a pulse of $Na_2HP^{32}O_4$ from 16 to 30 hr after a mock infection. The elution pattern of this mixture from the column is shown in Figure 3. The first peak to elute from the infected culture corresponding to the position of viral DNA is not present in the control culture. The surprising finding is that about two thirds of the DNA synthesized in the infected culture elutes late, in a position corresponding to that of cellular DNA.

In order to test whether the late-eluting DNA is cellular, rather than a viral DNA with unusual elution properties, experiments of DNA-DNA hybridization with mouse kidney DNA were performed. A large quantity of DNA was isolated from an infected culture which had been labeled with H^3-thymidine 16–30 hr after virus adsorption; this DNA was then chromatographed on a column of methylated albumin. The fractions were tested for infectivity. The elution pattern from the column and the infectivity of each fraction are presented in Figure 4. It can be seen that infectivity is present only in the early eluting peak. Three fractions from the early peak were combined (*A*) as were three fractions from the late peak (*B*). These were exposed to intense sonication, and tested for their ability to hybridize with mouse kidney DNA by the agar-column method.[7] In the first experiment, samples from *A* and *B* with approximately the same amount of radioactivity were hybridized with mouse kidney DNA. As shown in Table 2, experiment 1, the efficiency of hybridization of the DNA from peak *B* was 10 times greater than that of the DNA from peak *A*. Fractions *A* and *B* differ in total DNA content, since peak *B* contains all the pre-existing cellular DNA. This difference was eliminated in a second experiment by adding excess cellular DNA to both samples; they were then tested for their ability to hybridize with mouse kidney DNA. As shown in Table 2, experiment 2, the DNA of sample *B* again hybridized 10 times more effectively than that of sample *A*. The maximum extent of hybridization with homologous DNA was determined by hybridizing H^3-labeled DNA from uninfected cells with the same DNA preparation of mouse kidney. The extent of hybridization was similar to that of the DNA of peak *B*, as seen in Table 2, experiment 3. This shows that the DNA of peak *B* is mostly cellular DNA.

Two points thus emerge from the study of DNA synthesis in the virus-infected cells: (1) the total rate of synthesis is increased by a factor ten over that of the

control cells; (2) most of the DNA synthesized is cellular. We therefore conclude that the virus induces the synthesis of cellular DNA.

Time course of viral and cellular DNA synthesis: The times and rates of synthesis of total DNA and of infectious viral DNA in the infected cells are markedly different, as seen in Figure 1. To see whether the appearance of infectious DNA reflects the synthesis of viral DNA molecules, the following experiment was carried out. The DNA of separate cultures pulse-labeled with H^3-thymidine between 20 and 22, 22 and 24, and 30 and 32 hr after infection, was fractionated in a column of methylated albumin. The ratios of cellular to viral DNA, estimated from the elution patterns, showed that the incorporation of radioactivity into viral DNA molecules corresponds to the appearance of infectious DNA.

Rate of RNA and protein synthesis: No large differences were observed in the rates of incorporation of H^3-uridine into total cell RNA or of H^3-leucine into total cell protein of infected and noninfected cultures from 0 to 30 hr after infection.

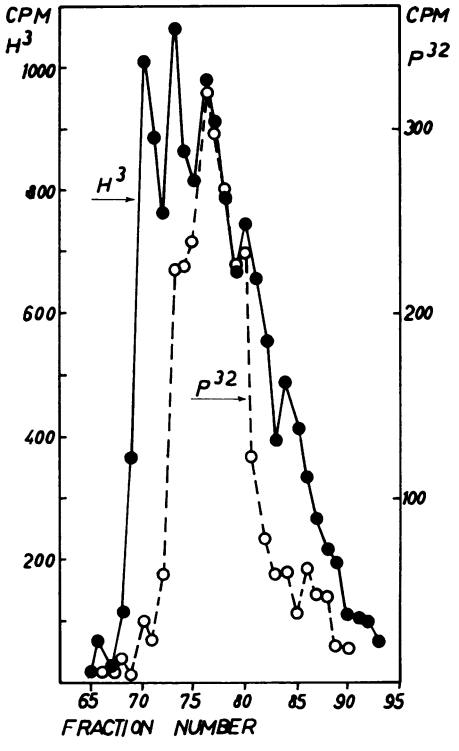

FIG. 3.—Fractions from a column of methylated albumin. The P^{32}-labeled DNA from uninfected cells (○- - -) and the H^3-labeled DNA from infected cells (● ——) are from the experiment described in the legend to Table 1. The total number of counts present in each fraction is given.

Changes in enzyme activities: The dramatic changes in the rate of DNA synthesis after infection prompted a study of the behavior of enzymes involved in DNA synthesis. The activities of the following enzymes were studied: thymidine kinase, deoxycytidine monophosphate-(dCMP-)deaminase, dCMP-kinase, deoxyadenosine monophosphate-(dAMP-)kinase, and DNA polymerase. All the enzymes studied retain the activity they have in the control cells until about 12 hr after infection. At this time, the activities of thymidine kinase, dCMP-deaminase, and DNA polymerase begin to increase (Fig. 5). Thus, the increase of the three enzyme activities follows the same time course until 28 hr, when a maximum is reached. The time at which 50 per cent of the increase is achieved is almost identical for the three enzymes. In the control cells the activities steadily decline.

The activities of dCMP and dAMP-kinase showed no changes. Thus, the pattern of changes of enzyme activities is similar to that observed in liver regeneration.[14]

Role of the capsid protein in the induction of the enzyme activities: In the infection procedure, each cell is exposed to thousands of virus particles; thus, the role of the viral protein in causing the observed effects must be evaluated. This was done by taking advantage of the separation of the virus into two bands upon equilibrium

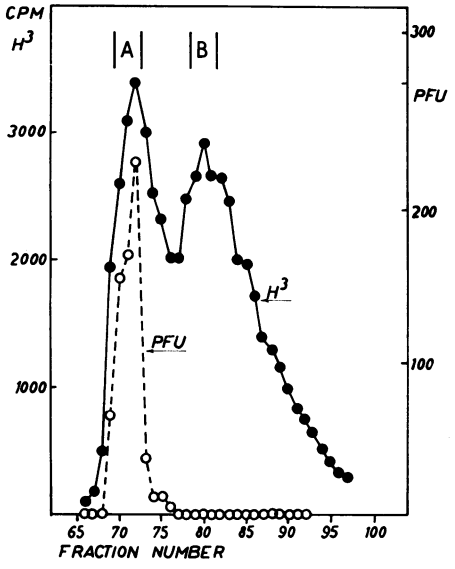

FIG. 4.—Fractions from a column of methylated albumin; same H^3-labeled DNA preparation of Fig. 3. H^3-labeled DNA (● ——); infectious DNA (○ - - -). Radioactivity is in cpm in 0.1 ml of each fraction, and infectivity is in PFU per ml.

density gradient centrifugation in CsCl: a bottom band containing complete virus particles and a top band containing primarily empty capsids. The bottom band and the top band were obtained from the same virus lysate; they had a comparable number of particles as judged from their hemagglutination titer. The infectious titer, however, was 7 times higher in the viral band; thus, the top band was contaminated with about 15 per cent infectious virus particles. Parallel cultures were infected with different amounts of the two virus preparations. Extracts of the cultures were made 24 hr after infection and assayed for thymidine kinase and dCMP-deaminase activities. The results showed that the activities of the cultures were related to the infectious and not to the hemagglutinating titers. Thus, the enzyme activities are induced by the infectious particles and not by the viral capsids.

Discussion.—The induction of cellular DNA synthesis appears to be a unique property of polyoma virus among the animal viruses studied thus far. For instance, studies with the DNA-containing vaccinia and pseudorabies viruses never showed stimulation but often inhibition of host DNA synthesis following virus infection.[15]

The property of polyoma virus to cause the enhancement of enzyme activity and DNA synthesis may well be related to the ability of polyoma virus to transform normal cells into tumor cells, for the following reasons.

The multiplication of mouse kidney cells in cultures is regulated by a control phenomenon based on population density; that is, when the cells have formed a monolayer in the Petri dish, the rate of DNA synthesis and mitosis decreases greatly, so that the cells do not pile up in a multilayered sheet. The cultures employed in these experiments had reached that stage and had, therefore, a very low rate of DNA synthesis. The infection of these cultures with polyoma virus caused the failure of the control phenomenon and induced the synthesis of new

TABLE 2

Expt.	Peak	Unhybridized DNA (cpm fractions 1–10)	Hybridized DNA (cpm fractions 10–15)	% Hybrid
1	*A*	6593	93	1.4
	B	4495	953	17.5
2	*A* + unlabeled cell DNA	7111	83	1.2
	B + unlabeled cell DNA	6396	1049	15.4
3	H^3-cell DNA	6850	1658	19.5

Hybridization of DNA from peaks *A* and *B* (Fig. 4) with mouse kidney DNA. Sample *A* contains about 1 μg of H^3-labeled DNA per ml. Sample *B* contains about 1 μg of H^3-labeled DNA plus 4 μg of unlabeled cell DNA per ml. In experiment 2, 10 μg of sonicated, heat-denatured cell DNA was added to each of the 0.3-ml aliquots of *A* and *B* before hybrid formation. In experiment 3, 10 μg H^3-labeled cell DNA was tested for hybridization.

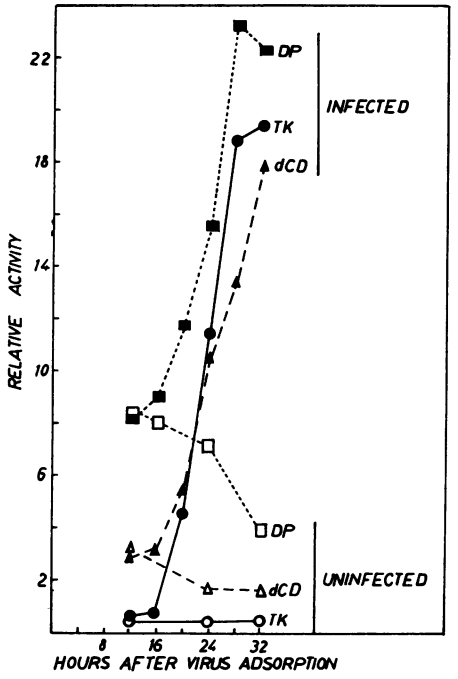

FIG. 5.—Activities of thymidine kinase (TK, O ——), dCMP deaminase (dCD, Δ - - -), and DNA polymerase (DP, □ . . .) as a function of the time after virus adsorption. Relative activities are proportional to the counts incorporated into products and normalized for protein content of reaction mixture.

cellular DNA. These cells are destined to die as a result of the virus infection; the loss of control, however, is directly analogous to that occurring in cells which are transformed by the virus. Cells transformed *in vitro* by polyoma virus become insensitive to control by population density; they give rise to dense multilayered cultures. The function which the virus performs to release the transformed cells from control may be the same function which the virus performs to induce uncontrolled cell-DNA synthesis in the cytocidal infection of mouse kidney cells. Thus, it may be possible to study events of major importance for cell transformation in a system in which the cells are not undergoing transformation but are being killed by the virus. This system offers a distinct advantage in that nearly all of the mouse kidney cells undergo the cytocidal infection while cell transformation occurs in a small proportion of the cells in the systems so far available.

Summary.—Crowded cultures of mouse kidney cells have a very low rate of DNA synthesis, and very low activities of the enzymes involved in DNA synthesis. After infection with polyoma virus, both the enzyme activities and the rate of DNA synthesis markedly increase. It is of special interest that the DNA synthesized in the infected cells is predominantly cellular. The ability of the virus to stimulate the synthesis of cellular DNA may be related to its tumorigenic property.

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ACYL CARRIER PROTEIN, IV. THE IDENTIFICATION OF 4′-PHOSPHOPANTETHEINE AS THE PROSTHETIC GROUP OF THE ACYL CARRIER PROTEIN

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In previous reports it has been shown that all of the reactions of fatty acid synthesis in *Escherichia coli* occur with the substrates bound in thioester linkage to the acyl carrier protein (ACP).[1–10] The nature of the substrate binding site of ACP is of great interest as several types of reactions occur with the substrates bound to this protein. Thus, thioesters of ACP are substrates in the condensation-decarboxylation reaction,[6, 7, 10] the 2 reductions,[7, 8] and the enoyl hydrase reaction of fatty acid synthesis.[9] In each of these reactions acyl-S-ACP derivatives are either much more reactive than the corresponding acyl-S-CoA compounds, or the latter do not react at all. Initially it was reported[6] that the sulfhydryl group at the binding site of ACP is a cysteine residue. Wakil[11] reported that the sulfhydryl residue is accounted for by thioethanolamine and that ACP contains one mole of β-alanine per mole of protein. These findings have been confirmed in this laboratory. Further investigation has shown that the sulfhydryl residue is part of a covalently bound prosthetic group. This report presents experiments which establish the binding site of ACP as 4′-phosphopantetheine which is probably bound through a phosphodiester linkage to a serine residue of ACP.

Materials and Methods.—2-C^{14}-Malonyl CoA was synthesized as described previously.[12] ACP was purified as described previously from either *E. coli* B or from *E. coli* K12.[6] Dowex resins were purchased from Calbiochem (Biorad). P^{32}-orthophosphate was obtained from Oak Ridge National Laboratory. 2-C^{14}-Malonic acid was purchased from the New England Nuclear Co. Pronase was purchased from Calbiochem. Pepsin 2 × crystallized and *E. coli* alkaline phosphatase