THE INCORPORATION OF LEUCINE-C^{14} INTO PROTEIN BY A CELL-FREE PREPARATION FROM MAIZE KERNELS

By Robert Rabson and G. David Novelli

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY,* OAK RIDGE, TENNESSEE

Communicated by Alexander Hollaender and read before the Academy, November 17, 1959

In investigations of the biochemical mechanisms involved in gene-induced changes in the enzymes that regulate starch biosynthesis[1] in maize endosperm, it would be desirable to have available a cell-free system from maize endosperm that is capable of synthesizing these proteins. The present communication is a report of attempts to develop such a system. Although net protein synthesis has not yet been achieved, we have found a preparation that actively incorporates radioactive leucine into protein. This preparation has many characteristics in common with the well-known microsomal system from rat liver.[2] A preliminary report of this work has appeared.[3]

Materials and Methods.—Immature ears of waxy maize in the late milk stage were obtained from the crossing field of Dr. Drew Schwartz of this Laboratory. After harvest, all handling was conducted in a cold room at 3°C. The ears were husked and the whole kernels removed with a razor blade or sharp knife. Kernels were ground in a manner similar to that described by Raacke,[4] about 100 g in a cold mortar with 100 g of grinding sand and about 90 ml of 0.45 *M* sucrose in 0.1 *M* potassium phosphate buffer, pH 7.5. The kernels were ground vigorously for about 5 minutes until only a few small fragments of unground pericarp were still obvious. After filtration through three layers of coarse cheesecloth, the homogenate was centrifuged for 10 minutes at 1,000 × *g* in a Lourdes refrigerated centrifuge. The pellet, containing starch and cell debris, was discarded and the supernatant was centrifuged for 15 minutes at 10,000 × *g*. The supernatant solution was carefully removed with a large syringe and the pellet discarded. This supernatant was centrifuged at 100,000 × *g* for 60 minutes. The supernatant was carefully removed and saved for later fractionation. The pellet was carefully swirled in 1 ml of distilled water until free from the tube and then gently homogenized and made to a volume of 12 ml with distilled water. Any insoluble material was removed by centrifugation at 10,000 × *g* for 15 minutes; the supernatant was used for most of the experiments. When "washed" particles were prepared, the suspension was centrifuged at 100,000 × *g* for 60 minutes; then the pellet was resuspended in distilled water and, after homogenization, was centrifuged at 100,000 × *g* for 60 minutes. Resuspension and centrifugation were repeated. Finally the pellet was resuspended, homogenized, and suspended in 12 ml of distilled water.

The pH 5.2 fraction was prepared from the original 100,000 × *g* supernatant by adjustment to pH 5.2 with 2 *N* acetic acid. The precipitate was collected by centrifugation at 10,000 × *g* for 15 minutes. The pellet and tube were gently washed with distilled water, and then the pellet was dissolved in 0.05 *M* potassium phosphate buffer at pH 7.4.

The individual incubation mixtures are given for each experiment in the accompanying tables and figures. Incubations were carried out at 37°C in 10-ml flasks, with shaking. Reactions were stopped by pipetting 1 ml of 10 per cent

trichloroacetic acid (TCA) into the flask. Immediately before this, 0.1 ml of 0.1 *M* nonradioactive leucine was added to the contents of the flask.

The TCA precipitates were washed with cold 5 per cent TCA, then twice with hot 5 per cent TCA (in boiling water bath for 10 minutes), and twice with an ethanol:ether mixture (1:1, v/v). The precipitates were dried in an oven at 80°C and then solubilized in 0.2 to 1.0 ml of 80 per cent formic acid. Aliquots were taken for counting radioactivity and for measuring protein by the method of Lowry *et al.*,[5] crystalline bovine plasma albumin being used as a standard. For counting, 0.1 ml of the solution was pipetted into a bottle containing 10 ml of the following mixture: 700 ml of toluene, 300 ml of absolute ethanol, 4 g of 2,5-diphenyloxazole, and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene, and the sample was counted in a Packard liquid scintillation spectrometer.

Results.—The data presented in Table 1 illustrate the energy requirement of the maize-incorporating system. They also show the stimulation of incorporation by GTP. Thus, when both the GTP and the ATP-generating system are omitted, very little leucine is incorporated into protein. Table 2 shows the dependence of the

TABLE 1

ENERGY AND GTP REQUIREMENTS FOR AMINO ACID INCORPORATION IN MAIZE PARTICLES

Incubation Mixture	Incorporation, cpm/mg of protein Experiment A	Experiment B
Complete*	200	143
Minus ATP	143	117
Minus GTP	166	92
Minus ATP generator	54	75
Minus ATP and GTP	...	18

* Consists of 50 μmoles of phosphate buffer (pH 7.4), 10 μmoles of $MgCl_2$, 1.0 μmole of ATP, 0.3 μmole of GTP, 6 μmoles of phosphoenolpyruvate, 20 μg of pyruvate kinase, 0.05 μmole of L-leucine-C^{14} (2.32×10^6 cpm). Expt. A: 2.5 mg of protein; expt. B: 2.7 mg of protein. Total volume, 1.0 ml. Incubation: 37°C, 60 minutes.

TABLE 2

EFFECT OF RNASE, CHLORAMPHENICOL, AND EDTA ON INCORPORATION IN MAIZE PARTICLES

Expt.	Incubation Mixture	Incorporation, cpm/mg of protein	Percentage Inhibition
C	Complete*	93.6	
	+ Chloramphenicol, 200 μg	69.1	27
	+ RNase, 400 μg	0.0	100
D	Complete	683.5	
	+ Chloramphenicol, 400 μg	261.7	61.6
	+ RNase, 400 μg	16.6	97.6
	− $MgCl_2$	20.7	96.5
E	Complete	356.0	
	+ EDTA, 20 μmoles	18.3	94.7
	+ EDTA, 40 μmoles	32.0	91.1

* As in Table 1, except: Expt. C—0.05 μmole of L-leucine-C^{14} (2.32×10^6 cpm), 1.2 mg of protein. Expt. D—0.05 μmole of DL-leucine-C^{14} (4.64×10^6 cpm), 0.9 mg of protein. Expt. E—0.05 μmole of DL-leucine-C^{14} (4.64×10^6 cpm), 1.0 mg of protein. Incubation: 37°C, 30 minutes.

system on Mg^{++}. These requirements are similar to those reported for the rat liver microsomal incorporating system.[2]

The time course of the incorporation process is shown in Figure 1. Incorporation is a rapid process and, like the comparable system from rat liver, the reaction is over in 30 minutes. There is no apparent explanation for the reaction's stopping at this time. In one experiment the particles were separated before the protein

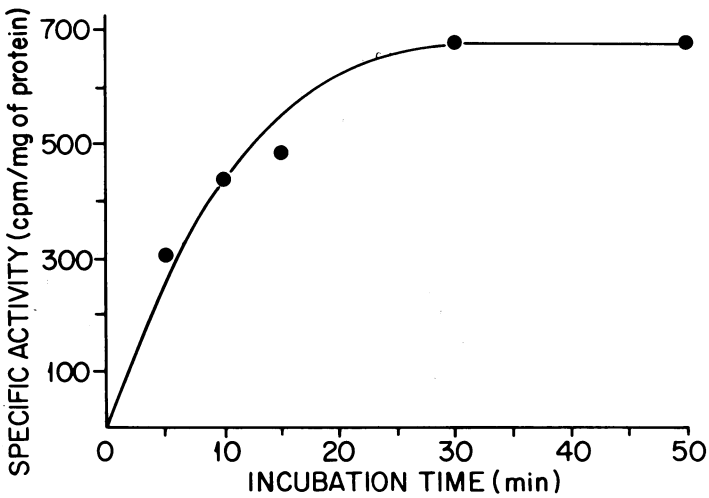

FIG. 1.—Time course of amino acid incorporation into maize particles. Potassium phosphate buffer; incubation conditions as for Table 1, except that 0.05 μmole of DL-leucine-C^{14} (4.64×10^5 cpm) was added.

was worked up for determination of radioactivity; 85 to 90 per cent of the incorporated leucine was still attached to the particles. This suggests that the particles may have a limited number of sites available for incorporation and perhaps the cessation of incorporation may be a consequence of filling the limited sites and the absence of an enzyme or factor to remove the newly formed protein.

As with all amino acid incorporation systems, we are always faced with attempting to distinguish between a process reflecting protein synthesis and one that may involve exchange reactions of one sort or another. In the absence of net protein synthesis, there is only indirect evidence for linking the incorporation system with protein synthesis. Such indirect evidence is presented in Table 2. It can be seen that the incorporation of leucine into maize protein is completely abolished by treating the system with ribonuclease. Chloramphenicol at 200 μg/ml caused a 27 per cent inhibition of incorporation and 400 μg/ml caused a 62 per cent inhibition. Because chloramphenicol is known to inhibit protein synthesis in bacteria, inhibition of incorporation of amino acids by chloramphenicol is often used as an argument that the incorporation process is a reflection of protein synthesis. Such an interpretation becomes questionable when the chloramphenicol concentration required to inhibit the incorporation process is 10 to 20 times (as in the present case) as great as that required to inhibit protein synthesis in bacteria. The recent observation that chloramphenicol at 2,200 μg/ml (100 times the concentration required for bacteria) inhibits the incorporation of amino acids into calf thymus nuclei[6] and leads to the accumulation of amino acids bound to RNA—the same point of inhibition of bacterial systems[7]—suggests that inhibition by chloramphenicol, regardless of concentration, may indeed reflect inhibition of protein synthesis.

The maize particle system seems to be more stable to storage than mammalian particles. The data of Table 3 show that particles can be stored at 3°C for 18 hours without much loss in activity, making it possible to work for several days with the same preparation. If particle preparations are frozen in liquid nitrogen,

they immediately lose about 50 per cent of their activity, but there is no further loss in activity on storage at liquid nitrogen temperatures for as long as 2 months. We have also observed that whole ears frozen at −20°C for as long as 1 week still

TABLE 3

EFFECT OF STORAGE ON PARTICLES AT 3°C ON INCORPORATING ABILITY

Experiment	Time of Storage	Incorporation, cpm/mg of protein
1	None	56.4
	18 hours	50.5
2	None	683.5
	18 hours	307.0
	1 week	48.5

Conditions as in Table 1, except: Expt. 1—L-leucine-C^{14}, 2.32 × 10^5 cpm. Expt. 2—DL-leucine-C^{14}, 4.64 × 10^5 cpm.

yield active preparations. On the other hand, particle preparations made from kernels stored at −20°C for 2 to 3 weeks are completely inactive. The age of the ear at the time of harvesting seems to be important in obtaining active preparations. Although the relation between age and active particles has not been completely worked out, our present information suggests that more-active preparations are obtained from ears harvested in the later stages of growth.

The experiments reported thus far were carried out with unwashed particles that did not require supplementation with any cytoplasmic component, although, as seen in Table 1, they did require a source of energy and GTP. It was of interest to determine whether the maize endosperm particles were like the pea seedling[8] particles that did not require supplementation with cytoplasmic constituents or whether they were like the liver microsomes that require the pH 5.2 fraction of the supernatant for full activity. Accordingly, the particles were washed as indicated in the method section and tested for requirements. The results of one such experiment are given in Table 4. It can be seen that washing the particles

TABLE 4

INCORPORATION BY WASHED MAIZE PARTICLES SUPPLEMENTED WITH pH 5.2 PREPARATIONS

Particles	pH 5.2 Preparation	Incorporation, cpm/mg of protein	Percentage of Control
Unwashed	None	484.8	100
Washed	None	24.5	5.3
Washed	Maize, 1.2 mg	379.2	78.2
Washed	Maize, 1.2 mg + amino acid	172.5	35.5
Washed	Maize, 4.8 mg	602.0	124.6
Washed	Rat liver, 0.4 mg	390.2	80.2
Washed	Rat liver, 1.6 mg	799.2	165.0
None	Maize, 4.8 mg	17.6	3.6

Complete system as in Table 1, except: DL-leucine-C^{14}, 4.6 × 10^5 cpm.

results in a loss of 95 per cent of their original activity. This activity can be largely restored by adding back a pH 5.2 fraction from either maize or rat liver. The best incorporation with 1.6 mg rat pH 5.2 represents about an incorporation of about 1.8 per cent of the added isotope. In this respect the efficiency of the maize system is comparable to the rat liver system.[2] On a protein basis, rat liver (at pH 5) seems to be superior to the comparable fraction from maize supernatant. The reason for this is not known, but it may be caused by different proportions of soluble RNA in the two preparations. Table 4 shows that a com-

plete mixture of amino acids resulted in a large suppression of the incorporation. The reason for this effect is also unknown. It may be caused by an imbalance of amino acids since, in a cell-free preparation from *E. coli*, a balanced mixture of amino acids that reflect the composition of *E. coli* protein is superior to an equimolar mixture of amino acids.

Summary.—A system from developing maize endosperm consisting of washed particles plus a pH 5.2 fraction from the supernatant that actively incorporates leucine-C^{14} into protein is described. The system requires a source of energy and GTP. The incorporation is inhibited by treatment with ribonuclease or chloramphenicol. When operating optimally, the incorporation efficiency is 1 to 2 per cent of the added isotope making this system comparable to the mammalian systems.

* Operated by Union Carbide Corporation for the U. S. Atomic Energy Commission.

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*REPRESSION OF AN ACETYLORNITHINE PERMEATION SYSTEM**

BY HENRY J. VOGEL

INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY

Communicated by Michael Heidelberger, February 29, 1960

In 1952, it was reported[1] that an *Escherichia coli* mutant (blocked early in the ornithine-arginine pathway[2, 3]), after preliminary cultivation on arginine, will grow without lag on ornithine or arginine, but with a pronounced lag on an ornithine precursor, subsequently identified[2, 4] as N^{α}-acetyl-L-ornithine. A study of this lag led to the observation[5] in 1953 that in wild-type *E. coli*, arginine antagonizes, i.e., represses,[6, 7] the formation of acetylornithinase (which converts acetylornithine to ornithine[2, 4, 8]). Similar instances in other amino acid pathways were described in the same year.[9]

Further investigations with the *E. coli* mutant and a derivative thereof have shown that, on suitable mixtures of arginine and acetylornithine, these organisms give diphasic growth: in the first phase, arginine is utilized preferentially at wild-type growth rate, and in the second phase (after exhaustion of the added arginine), acetylornithine is utilized at a slower growth rate.[5, 6] Analysis of this diphasic growth behavior has now provided evidence for the existence of a repressible acetylornithine permeation system.

Materials and Methods.—Organisms: The following strains of *Escherichia coli* were used: mutant strain 39A-23,[1] which gives a growth response to acetylornithine, ornithine, or arginine; 39A-23R2, a reisolate of 39A-23; W (ATCC 9637),