the cell, or the reading of the DNA molecules for RNA synthesis or replication is undisturbed by the breaks.

The behavior of infectivity at alkali pH suggests that single-stranded rings are infectious; only further experiments may, however, decide whether this is true.

Two results remain to be explained: (1) the difference in sedimentation velocity of the ring and of the linear molecules is larger than for other ring-shaped molecules, such as those of ϕX DNA[12] or λ DNA;[16] (2) the ring is unstable, as suggested by the kinetics of DNAase digestion. It can be tentatively suggested that they are the consequence of the combination of double strandedness and of the size of the ring.

We are grateful to Prof. R. L. Sinsheimer for useful discussions. The competent and dedicated assistance of Miss Maureen Muir is gratefully acknowledged.

This work will be supplemented by studies with the analytical ultracentrifuge.[17]

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*THE ROLE OF PROTEIN AND NUCLEIC ACID SYNTHESIS IN THE DEVELOPMENT OF RESPIRATION IN POTATO TUBER SLICES**

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The "wound respiration" of potato tubers has been under investigation for more than 75 years,[1] and attention has recently been focused on the changes which take place when thin slices of this tissue are maintained aerobically. Within a day after cutting, there are striking quantitative and qualitative changes in the respiration: the rate of oxygen consumption increases approximately fourfold, and the respiration becomes relatively insensitive to carbon monoxide and cyanide.[2] Some of the factors which influence these changes have been investigated,[3] but the under-

lying mechanisms which control them are not well understood. Although potato tuber slices are known to be able to synthesize proteins,[4] no causal relationship between synthesis and the rapid development of the respiration has been established. The experiments described in this paper were designed to answer the following question: does the increase in respiratory rate which takes place during the aerobic incubation of potato tuber slices depend specifically on the synthesis of new proteins and nucleic acids? It has been difficult to answer this question in the past, since specific inhibitors of these processes have not been generally available. Although it has been reported that chloramphenicol, an effective inhibitor of protein synthesis in bacterial systems, can prevent the respiratory increase in potato slices,[5] no evidence that this inhibitor was actually blocking protein synthesis was presented, and the high concentration (1 mg/ml) of chloramphenicol used raised the possibility of a general toxic effect. We have found that chloramphenicol at this concentration causes a marked loss of turgidity in the tissue, and its effect on respiration is not always reproducible. In view of the ambiguity of these results, we have investigated the effects of other specific inhibitors. It is shown here that low concentrations of puromycin and actinomycin D completely prevent the development of the rapid respiration in potato slices by inhibiting protein and nucleic acid synthesis in this tissue.

Materials and Methods.—Monthly shipments of potato tubers (*Solanum tuberosum*, variety White Rose) were obtained through the courtesy of Dr. H. Timm, Department of Vegetable Crops, University of California (Davis), and stored at 21°C. Cylinders of tissue were removed from a tuber with a cork borer (1 cm diameter), and 1 mm slices were cut using a hand microtome. The slices were washed for 25 min by stirring in sterilized, deionized water and either used immediately ("fresh" slices) or maintained at 21°C in small (6 × 1.5 cm) Petri dishes. To maintain the slices aerobically, they were placed on a piece of filter paper supported by 5 mm glass beads in such a way that they just broke the surface of the liquid. Each dish contained 11 slices and 8 ml of a solution containing 0.02 *M* phosphate buffer (pH 6.7), $5 \times 10^{-3} M$ $MgCl_2$, and $5 \times 10^{-4} M$ $CaSO_4$; 50 μg/ml of dihydrostreptomycin sulfate, which has no effect on the respiratory changes, was included to decrease bacterial contamination. All glassware was sterilized between experiments.

To follow protein synthesis, the incorporation of leucine-1-C^{14} into the slices was measured at appropriate intervals. The slices were removed from the radioactive solution and washed in 25 ml of nonradioactive leucine (0.2 mg/ml) for 30 min to remove any readily exchangeable leucine-C^{14}. They were then extracted with 70% ethanol for 8 hr in a Soxhlet apparatus, after which they were rinsed briefly with absolute ethanol. The alcohol-insoluble residue was dried at 80°C and counted directly in an automatic gas flow apparatus (Nuclear-Chicago) with an efficiency of approximately 5%. The alcohol-soluble material was counted in a Packard Tri-Carb scintillation spectrometer with an efficiency of 55%. To determine the fate of the label, aliquots of the alcohol-soluble material and the hydrolysate (6 *N* HCl, 100°C for 18 hr) of the insoluble residue were chromatographed on Whatman No. 4 paper using a methanol:H_2O:pyridine (80:20:4) solvent mixture, and the chromatogram was scanned with a Vangard strip chart counter. The only radioactive compound that could be identified in these fractions was leucine. When the alcohol-insoluble residue was ground to a powder and treated with ninhydrin,[6] no radioactivity could be detected in the CO_2 released.[7] This shows that the carboxyl groups of the radioactive leucine in the insoluble fraction were not free; they were presumably linked in peptide bonds.

Ribonucleic acid synthesis was followed by measuring the incorporation of uracil-2-C^{14} into the slices. The readily exchangeable uracil-C^{14} was first removed by washing for 30 min with 0.16 mg/ml uracil. The procedure used to extract soluble leucine did not remove all the soluble nucleotides, so an alternative procedure was developed. The slices were frozen and extracted with 10–20 ml of 60% boiling ethanol for 10 min; this procedure was repeated 5 times, after which the tissue was extracted twice with absolute ethanol. The radioactivity in the soluble fraction and in the insoluble residue were determined as described above for leucine. To determine the nature of the alco-

hol-soluble radioactive components, this fraction was made up to 95% in ethanol and 0.2 *M* in NaCl and allowed to stand for an hour at 0°C. The precipitate which formed was collected, washed, and counted; it contained less than 1% of the radioactivity in this fraction, suggesting that little, if any, RNA had been extracted. When an aliquot of the soluble fraction was subjected to paper electrophoresis in 0.2 *M* ammonium formate buffer (pH 3.7), four radioactive bands were separated and identified by the strip chart counter. Two of these bands corresponded to uracil and UMP, but the other two, lying between them on the paper, were not identified. The radioactivity which remained in the tissue after the alcohol extraction was almost certainly in RNA, since incorporation into this fraction was completely blocked by actinomycin. To obtain direct evidence that the uracil-C^{14} was incorporated into RNA, it was isolated using a modified phenol method.[8] The RNA was analyzed by the sucrose gradient technique,[9] and the profiles of the ultraviolet-absorbing material and the radioactivity were identical. The RNA obtained from actinomycin-treated tissue contained no radioactivity.

Respiratory rates were measured using standard Warburg manometric techniques. The nitrogen content of the alcohol-insoluble fraction remaining after the Soxhlet extraction was determined by the Kjeldahl method, using red mercuric oxide as the catalyst.

Actinomycin D was a generous gift from Merck, Sharp and Dohme, and chloramphenicol was donated by Parke, Davis and Company.

Results.—The general kinetic characteristics of the system under investigation are shown in Figure 1. The respiratory rate of the potato slices changed very little during the first three hr, after which it increased rapidly until it reached a plateau at approximately 15 hr. Our attention will be focused on the 24-hr period during which the rate of respiration increased approximately fivefold. The amount of leucine-C^{14} incorporated into the alcohol-insoluble fraction also increased rapidly after an initial lag period, and evidence that this incorporation corresponds to protein synthesis has already been presented (see *Methods*). Direct measurements of the nitrogen content of the alcohol-insoluble fraction showed that during this period the amount of insoluble nitrogen in the slices increased roughly parallel to the change in leucine incorporation. It seems likely that the amount of leucine incorporation was limited by the amount of radioactive leucine taken up by the slices, since the kinetics of the two processes were very similar (Fig. 2). In any case, the results with leucine-C^{14} indicated that some protein synthesis had taken place within 2 hr after cutting the slices, i.e.,

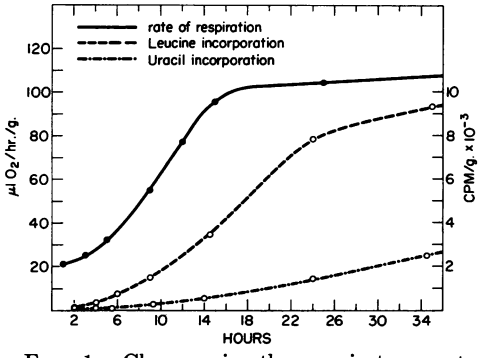

FIG. 1.—Changes in the respiratory rate, leucine incorporation, and uracil incorporation during the aerobic incubation of potato tuber slices. Solutions contained either 0.5×10^6 dpm leucine-C^{14} or 1.2×10^6 dpm uracil-C^{14} per 8 ml.

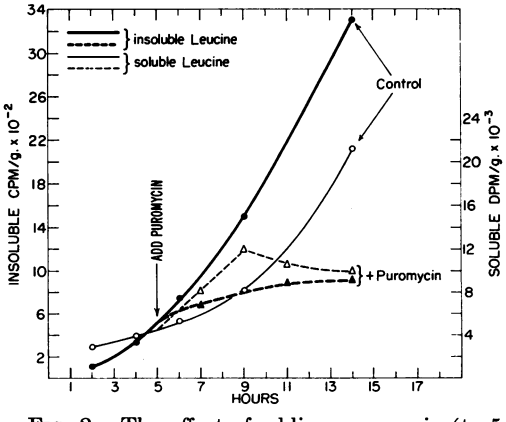

FIG. 2.—The effect of adding puromycin (to 5×10^{-4} M) after 5 hr on the uptake and incorporation of leucine (0.41×10^6 dpm per 8 ml).

TABLE 1

EFFECTS OF PUROMYCIN ON RESPIRATION AND INCORPORATION

Puromycin concentration (moles/liter)	Respiratory rate Q_{O_2}*	% Inh.†	Leucine incorporation‡ cpm/gm	% Inh.	Uracil incorporation§ cpm/gm	% Inh.
Control	120	...	9,930	...	1,770	...
0.6×10^{-4}	88	33	6,800	32	1,440	19
1.0×10^{-4}	77	49	4,350	55	1,230	31
2.0×10^{-4}	49	76	2,770	73	805	55
4.0×10^{-4}	29	97	650	93	356	79
8.0×10^{-4}	25	101	390	96	183	94

* Q_{O_2} = $\mu l O_2$/hr/gm.
† Per cent inhibition of the development of respiration (initial Q_{O_2} = 26 μl O_2/hr/gm)
‡ 0.51×10^6 dpm leucine-C^{14} per 8 ml.
§ 1.3×10^6 dpm uracil-C^{14} per 8 ml.

before there was a significant change in the respiratory rate.

The incorporation of uracil-C^{14} into the insoluble fraction started slowly and then increased steadily throughout the experimental period. The slow initial incorporation was not the result of a limitation imposed by the uptake of uracil-C^{14}, since the soluble fraction was found to contain 20,000 dpm and 78,000 dpm after 2 and 9 hr, respectively. It is more likely that this lag represented the time required for the conversion of uracil to uridine nucleotides that could be used for the synthesis of RNA. This conclusion is supported by the fact that the ratio of the amounts of UMP-C^{14} to uracil-C^{14} in the soluble fraction increased during the first 9 hr, but decreased in the subsequent 15 hr.

Effects of inhibitors: Puromycin is known to be an effective and specific inhibitor of protein synthesis in animal[10] and bacterial[11] cells. Table 1 shows the effects of maintaining potato slices for 24 hr in various concentrations of puromycin: at 4×10^{-4} M, the increase in respiration and the incorporation of leucine are essentially completely blocked. The effective concentrations of puromycin do not differ greatly from those used with other types of cells. There was a striking similarity in the degree of inhibition of the respiratory rise, on the one hand, and of leucine incorporation, on the other. Uracil incorporation was slightly less sensitive to puromycin than the other two processes, suggesting that the inhibition of RNA synthesis was a secondary effect.

The amount of leucine taken up by the slices during the 24-hr period was markedly inhibited by puromycin. This finding raises the following question: does the puromycin actually inhibit protein synthesis, or does it simply block the uptake of leucine-C^{14} into the cells and thereby prevent leucine-C^{14} incorporation? To test this possibility, 5×10^{-4} M puromycin was added to the slices *after* they had been maintained in a solution containing leucine-C^{14} for 5 hr, and the radioactivity in both the alcohol-soluble and -insoluble fractions was followed (Fig. 2). In the first few hr following the addition of the inhibitor, incorporation was severely inhibited, but the amount of leucine-C^{14} in the soluble fraction actually increased above the level in the control. This indicates that the primary effect of puromycin was on protein synthesis and not on the uptake of the amino acid. Further support for this conclusion was obtained from the demonstration that the increase in total nitrogen content of the alcohol-insoluble fraction was prevented by incubation of the slices for 24 hr in puromycin. Puromycin had very little effect on the total uptake of uracil-C^{14} or its conversion to UMP-C^{14}.

Puromycin appears to be a relatively specific inhibitor without deleterious side effects. It does not inhibit the rate of respiration. Incubation of day-old potato slices for 24 hr with a concentration of puromycin which completely prevented the development of the respiration did not affect the rate of respiration. Puromycin's effect on development is reversible. Freshly cut slices were kept in 5×10^{-4} M puromycin for 5 hr, removed, washed for 1 hr in distilled water, and then maintained in a puromycin-free solution. At the end of 24 hr (after cutting), the Q_{O_2} of these slices was only 31, but after an additional 24 hr it was 115. The latter rate was the same as that of the day-old controls, indicating that recovery was complete.

Actinomycin D, which inhibits nucleic acid and protein synthesis in bacterial[12] and animal[13,14] cells, completely prevented the development of the respiration in potato slices (Table 2). It also inhibited RNA synthesis completely, at concentrations of actinomycin which are as low as those effective in other systems. Protein synthesis was inhibited, but there was a basal level of leucine incorporation which was not eliminated by the highest concentrations of actinomycin used. When a correction was made for this actinomycin-insensitive leucine incorporation, the degree of inhibition of protein synthesis was found to correspond exactly to the degree of prevention of the respiratory rise. This suggests that only protein synthesis which is dependent on new RNA synthesis is linked to the development of the respiration.

TABLE 2

EFFECTS OF ACTINOMYCIN D ON RESPIRATION AND INCORPORATION

Actinomycin D concentration (μg/ml.*)	Respiratory rate		Leucine incorporation‡		Uracil incorporation§	
	Q_{O_2}*	% Inh†	cpm/gm	% Inh.	cpm/gm	% Inh.
Control	111	...	10,680	...	3,070	...
2.5	74	47	4,910	58	1,094	64
5.0	54	72	3,207	70	580	81
10	42	87	2,297	79	232	92
25	33	99	1,630	84	55	98
35	34	97	1,463	86	49	98

* Q_{O_2} = μl O_2/hr/gm.
† Per cent inhibition of the development of respiration (initial Q_{O_2} = 32 μl O_2/hr/gm).
‡ 0.69×10^6 dpm lecucine-C^{14} per 8 ml.
§ 1.5×10^6 dpm uracil-C^{14} per 8 ml.

There was no indication that actinomycin inhibited the uptake of either uracil or leucine, and it did not block nucleotide synthesis. The amounts of uracil and UMP in the alcohol-soluble fraction actually increased in the presence of this inhibitor. Actinomycin itself did not inhibit the respiratory rate at any time; for example, incubation of day-old slices for 24 hr in 25 μg/ml actinomycin did not affect the respiratory rate, even though RNA synthesis was prevented. This finding does not agree with the suggestion[15] that "the metabolic events which give rise to the respiratory increment must occur continuously for the rise to be sustained." Slices maintained in actinomycin showed no signs of injury and remained turgid throughout the experimental period. In order to determine whether slices which had been kept for a day in actinomycin still retained the characteristics of "fresh" slices, their response to dinitrophenol was studied. After 24 hr in 25 μg/ml actinomycin, the respiratory rate of the slices was exactly doubled by a brief exposure to 5×10^{-5} M dinitrophenol (pH 6.0); this stimulation corresponded exactly to the effect on "fresh" slices.[3]

A number of other compounds which have been widely used as inhibitors of protein and nucleic acid synthesis had no effect on the development of the respiration in potato slices, even when used at relatively high concentrations. These included: 5-fluorouracil (100 μg/ml), 5-fluorodeoxyuridine (200 μg/ml), 8-azaguanine (150 μg/ml), 5-methyltryptophan (1.5 mg/ml), and streptomycin (250 μg/ml). The results obtained with chloramphenicol (1 mg/ml) were varied, but when $MgCl_2$ and $CaSO_4$ were added to the incubation medium, this inhibitor did prevent the respiratory rise without apparent injury.

Effect of time of inhibitor addition: In order to clarify the time course and the sequence of the events underlying the respiratory change, inhibitors were added at intervals after the slices had been cut. For these experiments, concentrations of puromycin (5×10^{-4} *M*) and actinomycin (25 μg/ml) which completely prevented the respiratory rise when added initially were used. The results are shown in Figure 3; the length of time that the slices remained in the inhibitor, i.e., 24 hr minus the time of addition, is indicated on the abscissa. In all cases, the respiratory rates and amounts of radioactivity incorporated were determined 24 hr after the slices were cut.

It is clear from the Q_{O_2} determinations that increases in the respiratory rate were only prevented when the inhibitors were added within 8 hr after cutting the slices. The addition of actinomycin after one hr completely prevented the development. Additions after longer periods, but before 8 hr, resulted in partial inhibitions; for example, when actinomycin was added after $2^1/_2$ hr, the rate of respiration only doubled. On the other hand, when puromycin was added after $2^1/_2$ hr of aging, the development of the respiration was still completely prevented. These results indicate that the RNA synthesized in the first $2^1/_2$ hr after cutting is able to support a doubling of the respiratory rate, presumably by directing the synthesis of specific proteins; but if this protein synthesis is blocked, there is no increase in respiration.

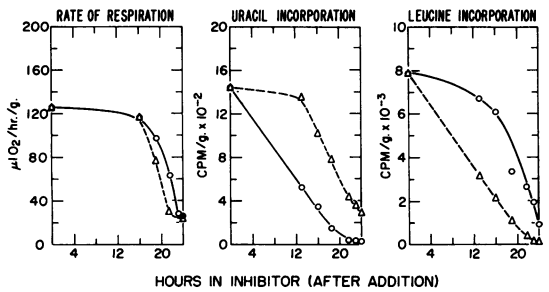

FIG. 3.—The effects of delayed additions of puromycin (- - -) and actinomycin D (——) to final concentrations of 5×10^{-4} *M* and 25 μg/ml, respectively, on the development of respiratory capacity, uracil (1.2×10^6 dpm per 8 ml) incorporation, and leucine (0.41×10^6 dpm per 8 ml) incorporation. The inhibitors were added after 0, 1, $2^1/_2$, 5, 8, and 11 hr incubation, and all measurements were made at the end of 24 hr.

A comparison of the kinetics of uracil and leucine incorporation with the effects of delayed inhibitor additions (Fig. 3) leads to the conclusion that actinomycin blocked uracil incorporation within an hr after its addition, and puromycin blocked leucine incorporation in less than 2 hr. The differences in the effects of the two inhibitors can be seen most clearly in the experiment where they were added after 11 hr of aging (i.e., 13 hr in inhibitor): actinomycin had little effect on the subsequent leucine incorporation but markedly inhibited the uracil incorporation, and puromycin had exactly the opposite effects. It will be recalled (Table 1) that, when puromycin was added initially, the uracil incorporation was blocked

TABLE 3

EFFECTS OF ADDING PUROMYCIN (TO 5×10^{-4} M) AND ACTINOMYCIN (TO 25 μG/ML) AFTER 5 HR OF INCUBATION ON RESPIRATION AND INCORPORATION DURING SUBSEQUENT 19 HR

Process measured	Per cent inhibition by Puromycin	Actinomycin
Development of respiration	67	38
Leucine incorporation	93	42
Uracil incorporation	49	97

more than 90 per cent; this indicates that RNA synthesis is more dependent on protein synthesis during the first few hr than in the later periods. The sensitivities of the respiration and the synthetic activities to the addition of inhibitors after 5 hr of aging are compared directly in Table 3. The per cent inhibitions were calculated on the basis of the changes which would have taken place in the subsequent 19 hr if the inhibitors had not been added. With puromycin, the percentage inhibition decreased in the following order: leucine incorporation, development of respiration, and uracil incorporation; with actinomycin, the sequence was: uracil incorporation, leucine incorporation, and development of respiration. These experiments indicate clearly that the primary effects of actinomycin and puromycin on the potato slices are on nucleic acid and protein synthesis, respectively, and they add further support to the view that the development of respiration depends on protein synthesis, preceded by RNA synthesis.

Concluding Remarks.—Previous work has shown that the rapid development of the "wound respiration" in potato tuber slices is entirely dependent on the general aerobic metabolism of the tissue.[3] During this period there are marked increases in the activities of a number of oxidative enzymes.[3, 16, 17] There are also rapid and striking changes in the path of carbon, leading to increased activity of both the pentose cycle[18] and the tricarboxylic acid cycle.[19] Our results indicate that all of these changes are dependent on the synthesis of new RNA and proteins. The control over these processes is probably exerted at the level of the cellular DNA, since this is the site at which actinomycin acts.[20] The nature of the specific trigger mechanism which permits the development of the respiration is not known, but synthesis of nucleic acid and protein must be released from some kind of repression soon after the slices are cut. Laties[21] has proposed that the development of respiration may result from the loss of a volatile inhibitor from thin slices of tissue, and has recently suggested that γ-hydroxy-α-ketoglutarate, an inhibitor of tricarboxylic acid cycle activity, may be involved.[22] Our results indicate that release from some respiratory inhibition will not *per se* account for the observed changes, since the development depends on the synthesis of new proteins.

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*AN EARLY EFFECT OF ESTROGEN ON PROTEIN SYNTHESIS**

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In studies of the mechanism of action of estrogens, Mueller, Gorski, and Aizawa demonstrated that, when protein synthesis was blocked by puromycin, the increases induced by estrogen in phospholipid and ribonucleic acid synthesis were also blocked.[1] They concluded that uterine responses to estrogen are dependent on protein synthesis and pointed out that the elucidation of the nature of this protein synthesis is critical to understanding the mechanism of action of estrogen. Estrogen may stimulate protein synthesis in general by influencing, directly or indirectly, some component of the protein-synthesizing systems of the cell. This would result in the nonselective increase in synthesis of various types of proteins, including those necessary for further expression of the estrogen response. Another possibility is that estrogen brings about the *de novo* synthesis of a limited number of proteins necessary for the uterine response to estrogen. These proteins might in turn influence various metabolic pathways, including the protein-synthesizing systems themselves. It was impossible to distinguish between these two possibilities previously, because studies of the estrogen effect on protein synthesis were carried out at time periods of 4 hr or greater after estrogen administration when the rate of over-all protein synthesis is markedly stimulated.[1, 2]

In order to ascertain the nature of the protein synthesis stimulated by estrogen, we investigated the effect of estradiol-17β on *in vivo* protein synthesis in various cell fractions at 2 and 4 hr after hormone treatment. The relationship between RNA and protein synthesis in rats treated 2 hr with estrogen was studied, utilizing the puromycin inhibition of protein synthesis.