THE SYNTHESIS OF RIBOSOMES IN *E. COLI*

I. THE INCORPORATION OF C^{14}-URACIL INTO THE METABOLIC POOL AND RNA

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ABSTRACT C^{14}-uracil is rapidly incorporated by *E. coli* at low concentrations. Approximately half the radioactivity passes directly into RNA with very little delay. The remaining half enters a large metabolic pool and later is incorporated into RNA. The total rate of uptake (growing cells) is not greater than the requirement for uracil and cytosine for RNA synthesis. The size of the metabolic pool is not influenced measurably by the external uracil concentration. No evidence is found for the existence of a fraction of RNA which is rapidly synthesized and degraded.

A. INTRODUCTION

As a necessary part of the quantitative measurements of the time course of incorporation of C^{14}-uracil into ribosomal RNA, reported in a succeeding paper (Paper III),[1] a study of the role of the metabolic pool has been carried out. The system has some novel features, for example, a large fraction of the C^{14}-uracil is directly incorporated into RNA without measurable delay while the remainder is equilibrated with a rather large pool of phosphorylated compounds.

These observations are of interest not only with regard to the way exogenous uracil is handled by the cell but also have significant implications for studies of metabolic pools in general. Therefore they are described separately in this paper and their implications for the mechanisms of pool formation and maintenance are discussed.

B. METHODS

All of the experiments were carried out with *E. coli* ML 30 growing exponentially in C medium (Roberts *et al.*, 1955) using maltose as a carbon and energy source. The temperature was 37°C and the generation time about 51 minutes. 2-C^{14}-uracil of specific activity

[1] For convenience, the many cross-references among this group of closely linked papers, published here together, will be written in this simplified style.

5 microcuries per micromole was obtained from the New England Nuclear Corporation. Membrane filters carrying thin samples of cells were counted in the tri-carb liquid scintillation counter (Paper III).

Measurements of total incorporation into cells and incorporation into TCA-precipitable RNA were carried out with the rapid filtering technique previously described (Britten, Roberts, and French, 1955). The ratio of radioactivities entering cytidylic and uridylic acids of RNA was determined by paper chromatography with the following solvent: tert-butyl alcohol, 12 N HCl, H_2O 70/6.7/23.3. For this purpose samples of whole cells washed in 5 per cent TCA were hydrolyzed overnight at 37°C in 0.380 N KOH. The KOH was then adsorbed on fine-grain carboxylic ion exchange resin and the supernatant placed directly on the chromatogram. Only insignificant amounts of radioactivity were observed except in the uridylic and cytidylic regions on the chromatogram.

C. RESULTS

1. *Major Features of C^{14}-Uracil Incorporation.* The results of an experiment in which 10^{-7} M C^{14}-uracil was supplied to cells are shown in Fig. 1. The uracil

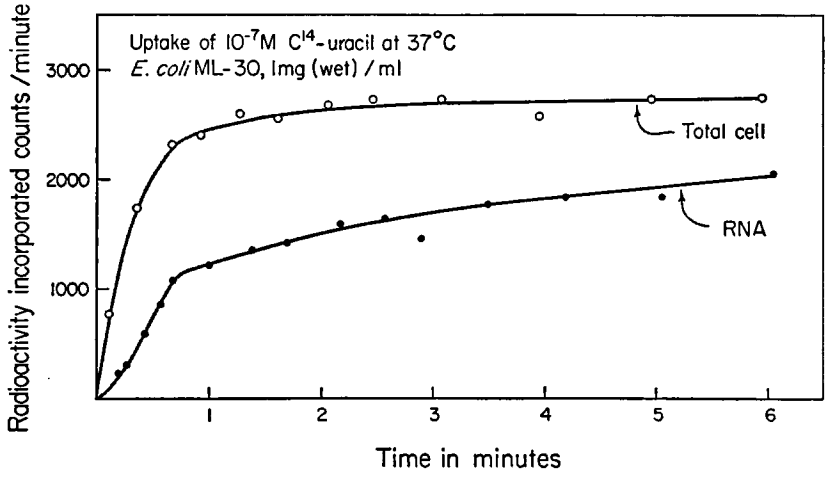


FIGURE 1 Incorporation of C^{14}-uracil into the metabolic pool and the RNA of *E. coli*. The difference between the two curves is the radioactivity in the metabolic pool. Initial uracil concentration was 10^{-7} M.

is almost completely removed from the medium by 40 seconds. During this period 2-C^{14}-uracil enters the RNA at a rate corresponding to about one-third of the total rate of uptake by the cells.

After 40 seconds when the C^{14}-uracil is completely removed from the medium, the rate of incorporation into RNA suddenly falls by a factor of about 5. During this second phase the rate of incorporation of uracil label falls slowly.

A semi-log plot made of the radioactivity of the pool as a function of time during this second phase shows an exponential decay with a time constant (decay to $1/e$) of about 9.0 minutes.

Fig. 2 shows the qualitatively similar results of an experiment at 10^{-6} M. The same two phases in the incorporation into RNA are observed. However in this case, the first phase, during which the C^{14}-uracil is still present in the medium, lasts for 2 to 3 minutes. The transition to the second phase is not quite so abrupt, but again an exponential decay of the pool radioactivity with a time constant of about 10 minutes is observed.

The basic properties of this system become clear when experiments at higher

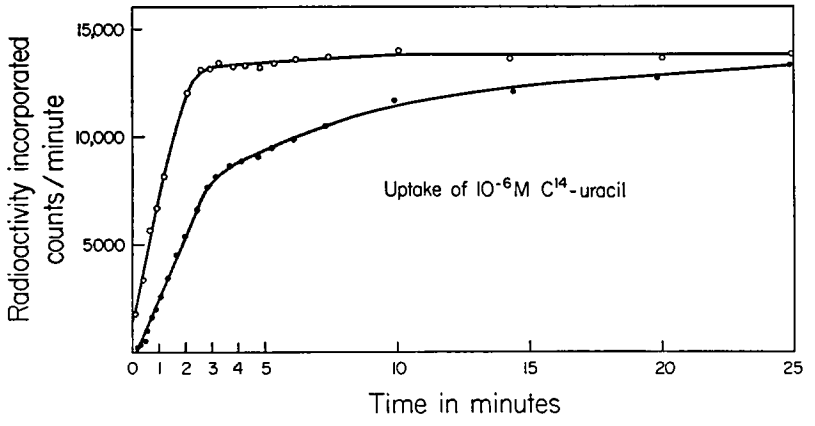
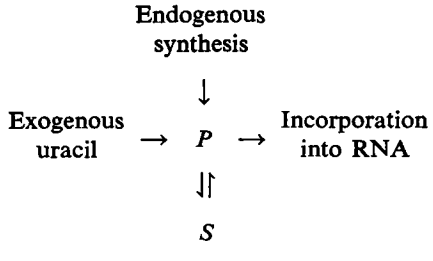


FIGURE 2 Incorporation of C^{14}-uracil into the metabolic pool and RNA of *E. coli* ML 30 at 37°C. Initial uracil concentration, 10^{-6} M. Cell concentration, 0.59 mg (wet) per ml. Open circles, total incorporation. Solid circles, incorporation into RNA.

concentrations are examined. Fig. 3 shows the incorporation of 2-C^{14}-uracil into RNA at 5×10^{-5} M and 10^{-3} M. In these cases the amount taken up into the pool is such a small fraction of the total uracil present that its determination by the difference between total and RNA is subject to great uncertainty and the data are not presented. However, the incorporation into RNA supplies the necessary information. Two phases in the curves are again observed; however, in these cases the rate of incorporation into RNA at later times is just greater than twice the initial rate.

A simple interpretation of these experiments is indicated in the following diagram:



This schematic diagram is presented here to define the symbols *P* and *S* in order to simplify the language used in later sections. *P* represents a very small pool or se-

quence of reaction steps leading from uracil to a chemical form suitable for incorporation into RNA. *S* represents a large pool of compounds which can exchange with some uracil compound in *P*. The rate of exchange between *S* and *P* is not fast, and equilibrium between the specific radioactivity of *P* and *S* requires several minutes, at least. *P* then effectively forms a bypass, around the large pool, for the entry of exogenous uracil into RNA.

2. *Lack of Expansion of the Pools.* The question must be raised as to whether the amounts of compounds in the states represented by *S* and *P* are

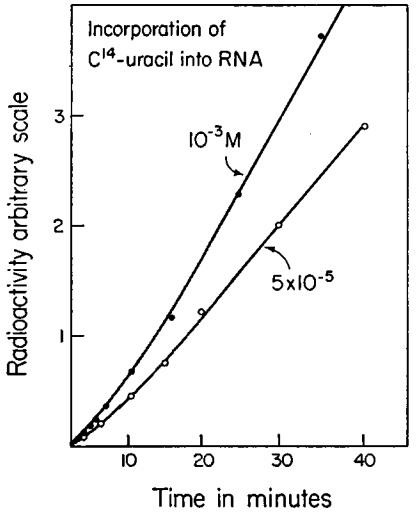


FIGURE 3 Incorporation of C^{14}-uracil into the RNA of *E. coli* ML 30 at 37°C. Solid circles, initial concentration 10^{-3} M. Open circles, initial concentration 5×10^{-5} M. The ordinate scales for the two curves are not related. The abscissa scale is proportional to increase in cell mass, with sample times indicated.

dependent on the concentration of exogenous uracil. That is, are these two pools expandable? This question is answered by an experiment (Fig. 4) in which C^{12}-uracil (4×10^{-5} M) was supplied to the cells for 10 minutes before the C^{14}-uracil was added. An identical curve within very small limits of error (< 5 per cent) was obtained in a simultaneously carried out control experiment in which an indentical quantity of C^{14}-uracil was added with the C^{12}-uracil at zero time. The concentration (4×10^{-5} M) is sufficient to achieve the maximum rate of entry of uracil into the cells. Therefore if the size of the two pools depended on the external concentration, a significant difference should appear between the control and the experiment in which C^{12}-uracil was added beforehand.

If *S* were expanded its specific radioactivity should rise rapidly in the control since the new material flowing in would be radioactive. On the other hand in the experiment in which the C^{12}-uracil was added beforehand the specific radioactivity should rise more slowly since a large amount of unlabeled uracil compounds would have been present in the pool at the time the C^{14}-uracil was supplied. It is evident that the pool *S* was not expanded by a measurable amount—certainly not by more than a small fraction of its preexisting size.

If any appreciable amount of unlabeled uracil compounds exists in state *P*, a delay in the entry of the radioactivity from exogenous uracil into RNA should be observed. There is no evidence of such a delay in the experiment of Fig. 4 after correction is made for the curvature due to the rising contribution of radioactivity from *S*. Without correction for this curvature the data for the first 5 minutes appear to extrapolate to a delay time of 10 seconds. The precision of the data and correction are such that the maximum actual delay time along the route through *P* must be less than 5 seconds.

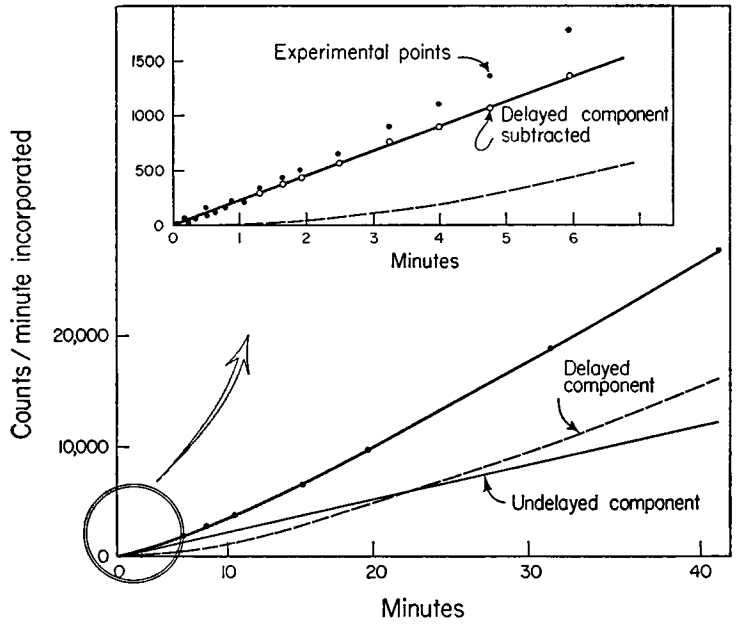


FIGURE 4 Incorporation of C^{14}-uracil into the RNA of *E. coli* ML 30 after 10 minutes' pretreatment with C^{12}-uracil at 4×10^{-5} M. The upper figure presents the data at early times with expanded scales. The light, solid, and dashed curves represent the best separation of the curve into a linear component and a component delayed by the pool (*S*). Solid circles are experimental values. For the open circles the component delayed by *S* was subtracted. The abscissa scale is proportional to increase in cell mass, with sample times indicated.

It can be concluded that the amount of compounds in the states represented by *P* is so small that it cannot be adequately measured by these techniques, and that it remains small in the presence of moderately high concentrations of uracil.

3. *Conversion of Uracil to Cytosine.* Previous experiments (Roberts *et al.*, 1955) have indicated that uracil is converted to a great extent to cytosine compounds by *E. coli*. The questions that arise now are: what is the rate of this process, and to what extent is the second phase in the incorporation into RNA influenced by this conversion?

TABLE I

CONVERSION OF URACIL COMPOUNDS TO CYTOSINE COMPOUNDS

Culture density	Sample A		Sample B	
	Time	C*/U*	Time	C*/U*
mg wet/ml				
2.8	16″	0.075	15′–00″	0.614
1.4	31″	0.100	15′–30″	0.618
0.70	1′–00″	0.184	16′–00″	0.605
0.35	2′–4″	0.230	17′–34″	0.650
0.175	4′–03″	0.280	19′–00″	0.625
0.087	8′–00″	0.392	23′–00″	0.675
0.044	16′–22″	0.570	31′–00″	0.663

1.1×10^{-7} M C^{14}-uracil was added to seven cultures at the cell densities listed in column 1. An aliquot of the culture was brought to 5 per cent TCA at the time listed in column 2. TCA was added to the remainder of the culture at the time listed in column 4. The ratio of radioactivity of cytidylic acid and uridylic acid was determined after washing, alkaline hydrolysis, and chromatography.

In the first experiment to be described, 2-C^{14}-uracil was supplied to growing cultures at a concentration of 1.1×10^{-7} M. In order to vary the period of time during which incorporation into RNA occurred and still achieve efficient utilization of tracer at early times, seven cultures were set up simultaneously at different cell densities. The cell densities were chosen so that the C^{14}-uracil in the medium would be nearly exhausted at the time sample A was taken, as shown in Table I.

As a control a part of each culture (B) was incubated an additional 15 minutes

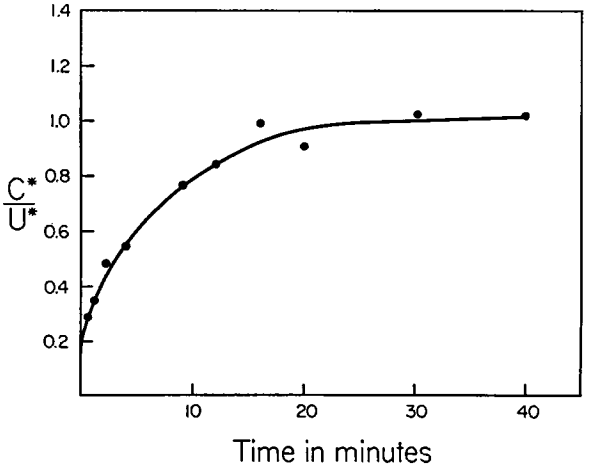


FIGURE 5 The ratio of the radioactivity of cytidylic acid to that of uridylic acid derived from the RNA of growing cells supplied 2×10^{-6} M C^{14}-uracil at zero time. Determined from chromatography after alkaline hydrolysis of samples taken into 5 per cent TCA.

TABLE II

RELATIVE RATES OF ENTRY OF C^{14}-URACIL INTO THE CYTIDYLIC AND URIDYLIC ACIDS OF THE RNA

Uracil concentration 2×10^{-6} M

	First phase 0 to 2 min.	Second phase 20 to 40 min.
Total	43	100
Uridylic	31	50
Cytidylic	12	50

so that almost all of the radioactivity which had entered *S* would be swept on into RNA. The ratio of radioactivity in cytidylic acid to that of uridylic acid in the RNA is almost constant in these samples. The slight increase is probably due to the fact that 15 minutes was not quite long enough to sweep all of the radioactive compounds out of *S*. This result is to be expected when there is not an induced (*i.e.* time-increasing) rate of conversion of uracil to cytosine, and implies that such an effect does not occur at this concentration of exogenous uracil.

The results shown in column 3 (Table I) indicate that uracil compounds are rather slowly converted to cytosine compounds and that after conversion the labeled cytosine compounds mix with a large endogenous pool of cytosine compounds before entering the RNA. The fact that some labeled cytidylic acid is observed in the RNA at the earliest time point suggests that there may be a small bypass around the pool of cytosine compounds.

On the basis of this experiment an estimate can be made of the kinetics of appearance of radioactivity separately in uridylic acid and cytidylic acid of the RNA. In the experiment at nearly the same concentration shown in Fig. 1, 40 per cent of the radioactivity was incorporated during the first phase. At the end of the first phase (at 40 seconds) the C*/U* ratio would be about 0.15 and at the end of the second phase about 0.66. Taking the total radioactivity incorporated into RNA as 100 per cent, we may then say: of the 40 per cent incorporated in the first phase, 35 per cent appeared in uridylic acid and 5 per cent in cytidylic; of the 60 per cent incorporated in the second phase 25 per cent appeared in uridylic and 35 per cent in cytidylic.

On Fig. 5 are shown the results of an experiment in which 2×10^{-5} M C^{14}-uracil was supplied to a growing culture of cells. Small samples were added to an equal volume of 10 per cent TCA, filtered, and assayed for radioactivity in order to measure the total incorporation. At several times larger samples were precipitated with TCA and the ratio of the radioactivity of the cytidylic and uridylic acids of the RNA was determined. The shape of the total incorporation curve was essentially identical to those shown in Figs. 3 and 4 and is therefore not reproduced.

The ratio of the radioactivity of the cytidylic to the uridylic acids of the RNA

is higher throughout the time course of this experiment than it was in the experiment at 1.1×10^{-7} M. Thus there is apparently a dependence of the conversion rate on the external concentration of uracil. This is not inconsistent with the lack of expansion of the pools. If the pools are in fact organized in the cell or under tight internal control, a considerable shift in chemical equilibria could occur without a measurable change in pool size.

From the data on Figs. 4 and 5 the rates of incorporation of radioactivity from uracil into the uridylic and cytidylic acids of the RNA may be calculated during the two phases. The results are given in arbitrary units in Table II.

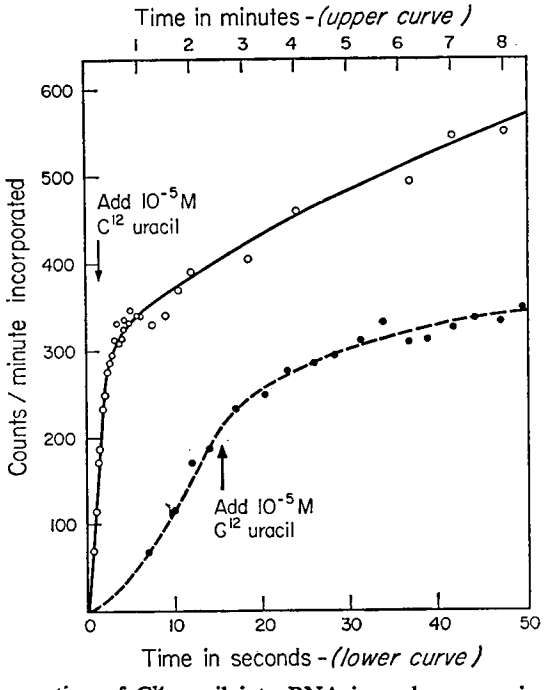


FIGURE 6 Incorporation of C^{14}-uracil into RNA in a chase experiment. 10^{-7} M C^{14}-uracil was added initially. At 15 seconds 10^{-5} M C^{12}-uracil was added. The lower curve (solid circles, dashed line) is identical to the upper curve but with an expanded time scale.

From these data it appears that there is an increase in the rate of incorporation into uridylic acid in the second phase. Moreover both experiments show that it is not purely the conversion of uracil compounds to cytosine compounds and delay by the pool of cytosine compounds which give rise to the second phase in the incorporation curves. This result is consistent with the existence of pools of UDP and UTP as well as CDP and CTP (Roberts, 1956). It also appears that there is some rapid conversion of uracil compounds to cytosine compounds since C*/U* ratio is already about 0.3 in the earliest point on Fig. 5. Therefore when C^{14}-uracil is supplied,

uracil and cytosine compounds become labeled both in states *P* and *S*, and conversion of uracil to cytosine may occur in both states.

4. *A "Chase" Experiment.* The word "chase" is used to represent a situation in which a labeled metabolite is present for a short period of incorporation and then followed by the incorporation of unlabeled metabolite.

Fig. 6 is an example of such a chase experiment. The cell density (1.7 mg wet/ml) is at the upper safe limit for steady exponential growth with good aeration for these cells. The initial C^{14}-uracil concentration (10^{-7} M) was the same as that of the experiment of Fig. 1. At this cell density, therefore, the first phase would be expected to last for about 25 seconds without a chase. The ratio of rates of incorporation of radioactivity into RNA before and after the chase was about 30 to 1.

The rate of incorporation after the chase is due to the quantity of radioactive compound which has entered the large pool, *S*. The specific activity of *S* is only slowly reduced by dilution after the chase. The relative entry of radioactivity into *S* can be minimized only by reducing the period of exposure to the tracer. Therefore with this system high ratios of the rate of incorporation into RNA before and after chase are achieved only with such short pulse times.

Such a short period of rapid incorporation followed by a sudden fall in the rate of incorporation is a favorable circumstance for the recognition of an RNA fraction which is rapidly synthesized and degraded. Such a fraction would be expected to fall rapidly in specific radioactivity very shortly after the chase. No fall in the TCA-precipitable RNA radioactivity is observed.

D. DISCUSSION

1. *The Pool of Uracil Compounds.* A striking result of these experiments is that external uracil is incorporated rapidly into RNA without delay even though there exists a large pool of uracil compounds which can also be incorporated into RNA. The implications of this result are represented on the schematic diagram. The meaning of the symbols *S* and *P* has been previously defined. This diagram not only suggests a possible mechanism for the bypass around the pool but also explains the detailed shape of the incorporation curves at high and low concentrations. The specific radioactivity of *P*, and therefore the rate of incorporation of radioactivity into RNA is the net result of the mixing of labeled uracil entering from outside with unlabeled uracil compounds internally synthesized and with uracil compounds from *S* which may be more or less labeled depending on the previous 15 minutes' history.

In the experiments of Figs. 1 and 2, as long as C^{14}-uracil enters the cell, radioactivity is incorporated into RNA at a relatively rapid rate. According to the diagram this is to be expected as long as exogenous uracil enters *P*. During this time radioactivity also enters *S*. When the C^{14}-uracil in the medium is exhausted the rate of incorporation abruptly falls to a new rate. Radioactive uracil compounds continue to flow from *S* into *P* and thence into RNA. The specific radioactivity of these com-

pounds and thus the rate of incorporation of radioactivity into RNA during the second phase will be determined by the amount of radioactivity that has entered *S* during the first phase. The relative rate of incorporation of radioactivity into RNA in the second phase is considerably higher in Fig. 2 than in Fig. 1. This is simply the result of the longer period during which radioactive uracil compounds have entered *S* yielding a higher specific radioactivity at the end of the first phase.

During the second phase the rate of entry of radioactivity slowly falls. In a number of experiments, the amount of radioactivity in the pool during the second phase (the difference between the total radioactivity in the cell and that in the RNA) was plotted on semilog paper. In every case a good straight line resulted and the time constant (decay to $1/e$) varied between 9 and 12 minutes. This is consistent with the diagram since the specific radioactivity of *S* should slowly fall as it is diluted by the flow of internally synthesized compounds.

In the experiments at higher concentrations (Fig. 3) the exogenous uracil lasts sufficiently long for the specific radioactivity of the uracil compounds of *S* to approach that of the uracil supplied. As a result the rate of entry of radioactivity into RNA slowly rises to its final value over a period of 10 to 20 minutes.

The ratio of the rate of flow into RNA to that into *S* is about the same in all of these experiments. However, the absolute rate of entry of uracil into the cell does increase with concentration, and reaches a saturation value not far different from the requirement for uracil plus cytosine for RNA synthesis. From a number of experiments the concentration at which the rate reaches half the saturation value appears to be about 2×10^{-7} M.

The quantity of compounds in the state *S* cannot be precisely evaluated from these experiments, due to the conversion of uracil compounds to cytosine compounds and to the existence of pools of both classes of compounds. Since the time constant of *S* is about 10 minutes and the flow through it just greater than half of the total flow into RNA, the total quantity of uracil and cytosine compounds can be roughly estimated to be the amount (of both classes) that is utilized for RNA synthesis in 5 to 8 minutes.

The best estimate of the quantity of uracil compounds in state *P* comes from the chase experiment (Fig. 6). The incorporation of C^{14}-uracil into RNA neither starts entirely abruptly nor drops to the second phase rate abruptly. The initial delay appears to be between 3 and 6 seconds and the time constant for the fall to the second phase rate appears to be about 7 seconds. The upper limit on the amount of uracil compounds in state *P* is therefore about the amount required for 5 seconds of total RNA synthesis or 0.1 per cent of the total uracil in the RNA. This must be considered an upper limit since there may be some delay in the entry of uracil into the cell.

The scheme proposed here to explain the observations on the incorporation of C^{14}-uracil has striking similarities to the "carrier model" (Britten and McClure,

1962) which has been proposed for the proline pools of *E. coli*. The experimental facts in the two cases differ in almost every conceivable way. The proline pool existing in the absence of supplement is very small; the maximum rate of uptake is 10 times the requirement for protein and the pool can be expanded to a very large size. There is no evidence of a bypass around the pool for entry of external proline into proteins. The only similarities are: both compounds are effectively taken up at low external concentrations; internal synthesis is effectively shut down at moderate concentrations; both compounds are incorporated into macromolecules.

However, it appears that by simply changing the values of a few of the reaction rate constants the predictions of the carrier model will be altered to agree with the observations presented in this paper on the incorporation of uracil. The reactions postulated in the carrier model are:

$$U + E \rightleftharpoons \overline{UE} \tag{1}$$

$$\overline{UE} + R \rightleftharpoons \overline{UR} + E \tag{2}$$

$$\overline{UE} \rightarrow RNA + E \tag{3}$$

$$\overline{UX} + E \rightarrow \overline{UE} + X \tag{4}$$

U symbolizes uracil compounds, ignoring all of the important chemical changes which must be carried out in a set of subsidiary reactions. E is the carrier, which is mobile within the cell and specific for the class of uracil compounds. R represents a set of specific storage sites. $\overline{UX}$ is the final reaction complex in the internal synthesis of uracil compounds. Uracil compounds are freed from it only through the reaction with free carrier, E. Thus equation (4) symbolizes the mechanism for control of the rate of internal synthesis. The reduction of the concentration of free carrier due to reaction with an excess of uracil will effectively shut down internal synthesis.

$\overline{UE}$ is the complex between the carrier and uracil compounds, and is equivalent to P in the schematic diagram. $\overline{UR}$ is the complex of uracil compounds with storage sites and is equivalent to S in the schematic diagram. If the equilibrium for reaction (2) is far to the right, then there will be little free R in any circumstance and a constant non-expandable pool (S) will result. If, in addition, the rates in reaction (2) are not fast, the observed slow equilibrium in specific radioactivity between S ($\overline{UR}$) and P ($\overline{UE}$) will result. If the dissociation constant in reaction (1) is small, the amount of free E will always be small and the rates in reaction (2) will hardly depend on the external uracil concentration, as observed. Further, the loss of uracil compounds will be small.

Reaction (1) represents the uptake of uracil into the reaction system. It is presumed that uracil can reach the carrier at a sufficient rate. Since the concentration of free E is probably small, it must have a high affinity for uracil.

Qualitatively, at least, the set of reactions (1), (2), (3), and (4) is consistent with the observations on the incorporation of uracil into the cell and into RNA.

These equations have been derived from the carrier model. However, they are sufficiently general that they may well be consistent with other models utilizing entirely different mechanisms. The ability of these equations to express compactly the characteristics of two such apparently different systems (uracil and proline) is both surprising and rewarding. It is suggestive that there is a kernel of truth in them.

2. *Absence of Evidence for Degradation of RNA.* The messenger hypothesis of Jacob and Monod (1961) proposes the existence of a small fraction of RNA which is synthesized, used as template for protein synthesis, and degraded. A rapidly labeled fraction of the RNA of bacterial cells has been observed (Bolton and Britten, 1958; Roberts, 1958; Britten *et al.,* 1959; Aronson *et al.,* 1960; Gros *et al.,* 1961). However, the rate of incorporation of labeled precursors into this fraction is exactly that which would be expected if it were a compulsory precursor to ribosomal RNA (Paper III). Hence its rapid labeling provides no proof of its degradation. Evidence for degradation must be obtained directly.

The delay in the labeling of ribosomal RNA shows that more than 90 per cent of the flow into ribosomes must pass through this fraction (Paper III). Hence, if it is not a compulsory macromolecular precursor to ribosomal RNA, the degradation products must be effectively reutilized. Consequently, if the synthesis and degradation can be represented as an exchange with an intermediate on the pathway for ribosomal RNA synthesis, the rate of exchange must be at least 10 times the flow to ribosomal RNA. The average lifetime of molecules in the fraction would therefore be less than 15 seconds, which seems too short for template material in view of the 2½ minute lifetime of the enzyme-forming unit for β-galactosidase (Pardee and Prestidge, 1961; Boezi and Cowie, 1961).

Further, if the hypothetical degradation products could mix with the metabolic pool of nucleotide precursors, then a fall in radioactivity of the RNA should be observed in the experiment shown in Fig. 6. No fall in radioactivity is observed after the chase, although at 15 seconds all of the RNA radioactivity must be present in the rapidly labeled fraction. Clearly, if any significant fraction of the degradation products entered *S* there would be an observable fall in RNA radioactivity since the product of specific radioactivity and rate of utilization of compounds from *S* is measured by the slow rate of incorporation into RNA after the chase. Equally, if the degradation products entered *P,* a certain fraction (probably more than half) would pass into *S,* since the kinetic experiments require that there be a significant circulation between the compounds of *P* and *S.* In this case, therefore, there should also be a fall in the RNA radioactivity.

Thus these experiments show no indication of degradation of the rapidly labeled fraction of RNA. Degradation could have escaped detection if only a small fraction of the newly made RNA were degraded or if the degradation proceeded only to the level of oligonucleotides. In this case the ribosomal RNA would be assembled almost completely from the "second hand" oligonucleotides. A third alternative is

that the proposed messenger is distinct from the rapidly labeled fraction. A small fraction of RNA which utilized precursors only from a slowly labeled pool such as *S* would escape detection in these experiments.

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