Sequential Translation of Trinucleotide Codons for the Initiation and Termination of Protein Synthesis

Abstract. *Terminal events in protein synthesis were studied with trinucleotide codons. Initiator and terminator trinucleotides sequentially stimulate* N-*formyl-methionyl-tRNA binding to ribosomes and the release of free* N-*formyl-methionine from the ribosomal intermediate. The release factor discovered by Capecchi is also required. The trinucleotides UGA, UAA, and UAG were found to be terminator codons. This pattern of codon degeneracy has not been observed with other trinucleotides and transfer RNA.*

Results of genetic (*1*) and biochemical (*2–4*) studies show that the synthesis of a peptide chain is terminated upon translation of the mRNA codons, UAA or UAG (*5*). More recently, UGA was also suggested as a barrier or terminator codon (*6, 7*).

The mechanism of peptide chain termination has been investigated by stimulating cell-free protein synthesis with randomly ordered polyribonucleotides (*8*), oligo- (*2*) or polyribonucleotides of known sequence (*9*), and viral RNA (*3, 4*). Translation of UAA or UAG results in the release of free peptides from ribosomes. The release of nascent peptide chains from ribosomes is dependent upon a partially purified release factor and the amber codon, UAG (*3*).

We now describe a simple, rapid assay for peptide chain termination, which is dependent upon terminator trinucleotides, the release factor, R, discovered by Capecchi (*3*), and the f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome complex. Incubation of reactions containing these components results in the release of free f[^{3}H]-methionine from ribosomes. Reactions then are adjusted to *p*H 1, f[^{3}H]-methionine is extracted with ethyl acetate (*10*), and the radioactivity of the ethyl acetate phase is determined. Reaction components and

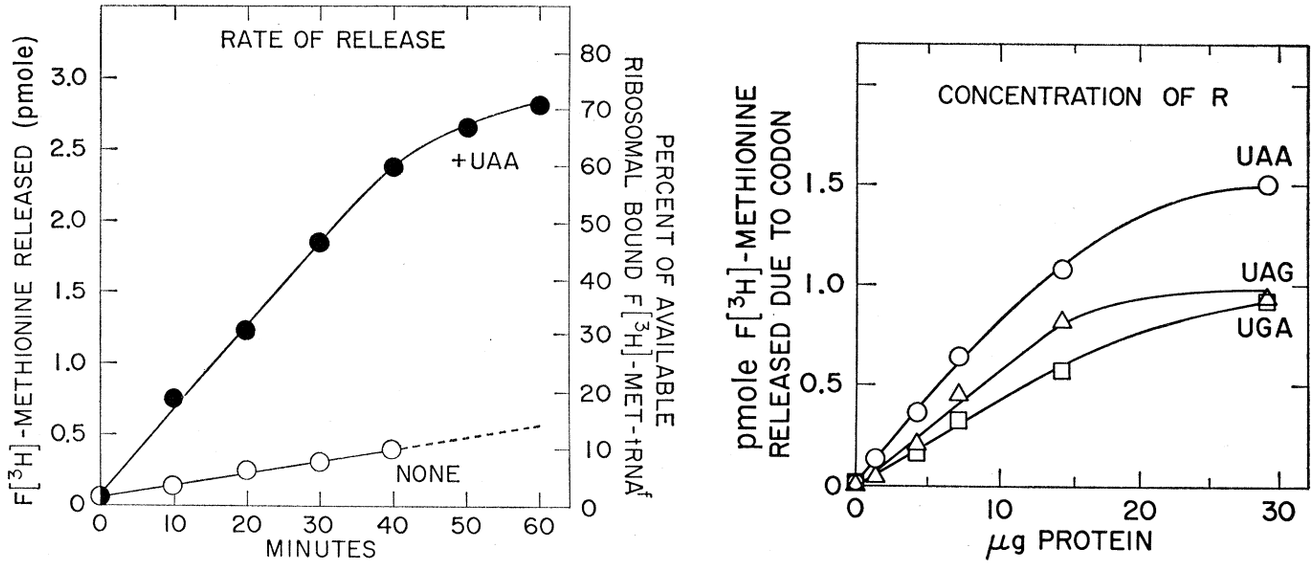

Fig. 1 (left). Formation of the f[^{3}H]-met-tRNA^f • • • AUG • • • Ribosome complex. Each reaction contained the following components in a final volume of 0.05 ml: 0.05*M* tris-acetate, *p*H 7.2; 0.05*M* potassium acetate; 0.01*M* magnesium acetate; 12 A^{260} units of *Escherichia coli* B ribosomes washed with 0.5*M* ammonium chloride (*16*); 76 pmole of f[^{3}H]-met-tRNA^f (0.23 A^{260} units) fractionated by benzoylated-DEAE column chromatography (*17*); and 2.08 nmole of AUG. Each reaction was incubated for 15 minutes at 30°C; then 0.20 ml of the following solution was added: 0.05*M* tris-acetate, *p*H 7.2; 0.05*M* potassium acetate; and 0.035*M* magnesium acetate (0.03*M* Mg^{++}, final concentration). The reaction was kept at 4°C prior to use in the termination assay. Usually 20 to 25 μl of the diluted reaction was added to a termination reaction. One A^{260} unit of the tRNA f-Met preparation accepted 330, 0.7, and 3.6 pmole of [^{3}H]-methyl-methionine (3.1 c/mmole), [^{3}H]-tryptophan (5.0 c/mmole) and [^{14}C]-cysteine (111 mc/mmole), respectively. *Termination reaction.* Each termination reaction contained the following components in a final volume of 0.05 ml unless otherwise stated: 0.05*M* tris acetate, *p*H 7.2; 0.05*M* potassium acetate; 0.03*M* magnesium acetate; 6.3 to 7.3 pmole of f[^{3}H]-Met-tRNA^f, 0.018 to 0.023 A^{260} units (4.8 to 6.2 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome complex); 0.96 to 1.2 A^{260} units of *E. coli* B ribosomes; 0.17 to 0.20 nmole of AUG; 5 to 40 μg of R protein; and, where indicated, 7.5 nmole of trinucleotide. Each reaction was incubated for approximately 15 minutes at 30°C, unless otherwise stated. Then 0.25 ml of 0.1*N* HCl and 1.5 ml of ethyl acetate were added, and the reaction was agitated for 15 seconds with a Vortex mixer. One milliliter of the ethyl acetate phase (top phase) was transferred to 10 ml of Bray's solution (*18*). The efficiency of extraction of f[^{3}H]-methionine into the ethyl acetate phase was 70 percent; the counting efficiency for the tritiated hydrogen was 10 percent. Corrections were applied so that each value corresponds to the amount of f[^{3}H]-methionine present in the entire reaction. Each point corresponds to a 0.05 ml reaction containing the components described above (including 3.78 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome complex and 0.78 μg of R protein, partially purified by DEAE-Sephadex column chromatography). The amount of f[^{3}H]-methionine present at zero times (0.30 pmole) was subtracted from each value (*22*).

Fig. 2 (right). Relation between the concentration of R and the rate of f[^{3}H]-methionine release from ribosomes. Each reaction contained the components described in the legend to Fig. 1, including 4.78 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome complex, the amount of R protein (prep S) shown above, and 7.5 nmole of either UAA (○), UAG (△), or UGA (□). The amount of f[^{3}H]-methionine formed without trinucleotides was subtracted from each value. The release factor was prepared from *E. coli* B, grown in minimal media and harvested during the mid-log phase of growth. Factor R (prep S) corresponds to a supernatant solution (after sedimentation at 137,000*g* for 3 hours) obtained by a previously described procedure slightly modified (*19*). Factor R (prep P) was obtained by precipitating R (prep S) protein with ammonium sulfate (0 to 70 percent), dissolving the pellet in buffer, and again precipitating protein with ammonium sulfate (0 to 35 percent). The pellet was dissolved in buffer, dialyzed, and stored in a liquid nitrogen refrigerator.

the method of assaying f[^{3}H]-methionine are described in the legend accompanying Fig. 1. R preparations are described in the legend to Fig. 2.

The effect of the trinucleotide UAA upon the rate of f[^{3}H]-methionine formation is shown in Fig. 1. The trinucleotide UAA markedly stimulated the release of f[^{3}H]-methionine. *N*-Formyl-[^{3}H]-methionine formation was proportional to time during the first 40 minutes of incubation; after 60 minutes of incubation, 72 percent of the ribosomal-bound f[^{3}H]-Met-tRNA^f present at zero time was converted to f[^{3}H]-methionine. For the other experiments described in this report, reactions were incubated for approximately 15 minutes; hence the values correspond to the rate, rather than the extent, of f[^{3}H]-methionine formation. Reproducibility of the assay was ± 10 percent.

The components required for UAA-dependent conversion of ribosomal-bound f[^{3}H]-Met-tRNA^f to free f[^{3}H]-methionine are shown in Table 1. Complete reactions contained f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome intermediates, UAA, R, and the other components described in the legend. Omission of UAA, R, ribosomes, or AUG from reactions, or incubation with 0.001*M* rather than 0.03*M* Mg^{++} reduced the rate of f[^{3}H]-methionine formation. The f[^{3}H]-Met-tRNA • • • AUG • • • ribosome complex is relatively stable at 0.01 to 0.03*M* Mg^{++} but dissociates rapidly at 0.001*M* Mg^{++}. Thus, the requirement for Mg^{++}, ribosomes, and AUG suggests that f[^{3}H]-Met-tRNA • • • AUG • • • ribosome intermediates are required rather than the dissociated components.

The UAA-dependent release of f[^{3}H]-methionine from the f[^{3}H]-Met-tRNA • • • AUG • • • ribosome complex is dependent on R. Cuzin *et al.* (*11*), DeGroot *et al* (*12*), and Rajbhandary *et al.* (*13*) have described an enzyme which catalyzes the hydrolysis of *N*-substituted aminoacyl-tRNA. The reaction catalyzed by this enzyme is not dependent upon ribosomes or terminator codons; hence, the enzyme differs from R.

Addition of unfractionated aminoacyl-tRNA, or of GTP and a GTP-generating system (phosphoenolpyruvate and phosphoenolpyruvate kinase), did not stimulate the rate of f[^{3}H]-methionine formation. In experiments not shown here f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome intermediates formed in the presence or the absence of initiation factors (*14*), GTP, and GTP-generating system were used to study UAA-dependent release of f[^{3}H]-methionine. The rate and extent of UAA-dependent f[^{3}H]-methionine formation found with both ribosome preparations were similar. On the basis of these studies initiation factors and GTP and a GTP-generating system were not included as standard reaction components.

Codon specificity is shown in Table 2. Trinucleotides UAA, UAG, and UGA stimulated the rate of f[^{3}H]-methionine

Table 1. Components required for codon-dependent termination. Each complete reaction contained the components described in the legend to Fig. 1 except where noted. In experiment 1, 6.24 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome were present per reaction (ribosomes or AUG were omitted where indicated) and 11.2 μg of R protein (prep P). In experiment 2, each reaction contained 4.2 pmole of f[^{3}H]-Met-tRNA • • • AUG • • • ribosome and 14.7 μg of R protein (prep S); where indicated, reactions contained 0.001*M* rather than 0.03*M* Mg^{++}. In experiment 3, each reaction contained 6.76 pmole of f[^{3}H]-Met-tRNA • • • AUG • • • ribosome; 14.7 μg of R protein (prep S); and where indicated, 0.250 A^{260} units of tRNA (*E. coli* B), 5 × 10^{-5}*M* each of 20 unlabeled amino acids, and 10^{-3}*M* ATP; 1 × 10^{-3}*M* GTP; 2 × 10^{-3}*M* phosphoenolpyruvate, potassium salt; and 0.25 units of phosphoenolpyruvate kinase (1 μg of protein). The amount of f[^{3}H]-methionine present at zero time (0.3 pmole) was subtracted from each value.

Reaction	f[^{3}H]-Methionine (pmole)
Experiment 1	
Complete	1.10
—Ribosomes	0.24
—AUG	0.49
—R	0.28
—UAA	0.45
—UAA, —R	0.24
Experiment 2	
Complete	1.46
—Mg^{++} (0.001*M*)	0.20
—UAA	0.47
—UAA, —Mg^{++} (0.001*M*)	0.18
Experiment 3	
Complete	1.20
Complete+tRNA+ATP+ 20 amino acids	1.21
Complete+GTP+PEP+ PEP kinase	1.20

Table 2. Specificity of codons for termination. Each reaction contained the components described in the legend to Fig. 1 including 5.04 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosomes; 11.2 μg of R protein (prep P); and 7.5 nmole of trinucleotide as indicated. The amount of f[^{3}H]-methionine present at zero time (0.35 pmole) was subtracted from each value. Preparation of trinucleotides, purity, and determination of base sequence have been described (*21*).

Addition	f[^{3}H]-Methionine (pmole)
NONE	0.40
UAA	1.06
UAG	1.07
UGA	0.93
CUU, AAU, or AGU	0.49
UUU, UUA, UGU, UGC, UGG, CUC, AAA, AUA, AGA, AGG, GUA, GAU	0.45

Table 3. Sensitivity of R to inactivation. In each experiment, 14.7 μg of R protein (prep. S) was incubated and then assayed for termination activity. In experiment 1, each reaction contained the components described in the legend to Fig. 1 (4.72 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome). Factor R (735 μg of protein) was incubated with 80 μg of trypsin (twice crystallized, containing no ribonuclease, Worthington) for 20 minutes at 30°C in a final volume 0.050 ml. Then 50 nmole of diisopropylfluorophosphate was added to inactivate the trypsin. For control reactions, R was incubated in the absence of trypsin under identical conditions, then trypsin that had been inactivated by diisopropylfluorophosphate was added. The amount of f[^{3}H]-methionine released in the absence of UAA (0.42 pmole) was subtracted from each value. In experiment 2, 735 μg of R protein was incubated with 0.01*M* *N*-ethylmaleimide for 15 minutes at 24°C in a final volume of 0.055 ml, then β-mercaptoethanol was added (0.1*M*, final concentration), and reactions were incubated for an additional 5 minutes before assay for termination activity. The R control was incubated in the presence of *N*-ethylmaleimide which had been inactivated with β-mercaptoethanol. The amount of f[^{3}H]-methionine released in the absence of UAA (0.85 pmole) was subtracted from each value. In experiment 3, 735 μg of R protein and 250 units of T_1 ribonuclease (Sankyo) were incubated for 20 minutes at 37°C in a final volume of 0.055 ml. The control was incubated under identical conditions in the absence of T_1 nuclease. The amount of f[^{3}H]-methionine released in the absence of UAA (0.72 pmole) was subtracted from each value. The R fraction (prep. S) contained endogenous tRNA; 0.20 and 0.80 pmole of [^{3}H]-threonine or [^{3}H]-glycine, respectively, were accepted by tRNA per 14.7 μg of R protein. Incubation of R with T_1 ribonuclease as specified reduced amino acid acceptance by tRNA by 95 percent. In experiment 4, 13.2 μg of R protein was added to each reaction. Where indicated, R was placed in a water bath at 100°C for 5 minutes, chilled, and then assayed for termination activity. Diisopropylfluorophosphate was added to termination reactions (10^{-3}*M*, final concentration) where indicated. Termination reactions were incubated for 10 minutes at 30°C. The amount of f[^{3}H]-methionine released in the absence of UAA (0.74 pmole) was subtracted from each value.

Experiment	Incubation of R	UAA-Dependent f[^{3}H]-methionine (pmole)
1	Control	1.08
	+Trypsin	0.05
2	Control	0.96
	+*N*-Ethylmaleimide	0.13
3	Control	0.73
	+T_1 ribonuclease	0.66
4	Control	0.56
	100°C	— 0.60
	Control + boiled R*	0.41
	Control + diisopropylfluorophosphate	0.56

* Added to termination reaction in addition to control R.

formation, whereas the other trinucleotides, including some with the same bases arranged in different sequence, had little detectable effect. Hence codon specificity is high.

Both UAA and UAG serve as terminator codons in vivo (*1*) and in vitro (*2, 3*). Genetic evidence reported by Brenner and co-workers (*6*) and Garen and co-workers (*7*) indicates that UGA serves either as a barrier or as a terminator of protein synthesis. Since f[^{3}H]-methionine is released from ribosomes in response to UGA, this codon, and also UAA and UAG, can serve as terminator codons.

The relation between the concentration of R and the rate of f[^{3}H]-methionine release from ribosomes is shown in Fig. 2. The results demonstrate that R is required for UAA-, UAG-, and UGA-dependent formation of f[^{3}H]-methionine, and that the rate of f[^{3}H]-methionine formation is proportional to protein in the R preparation within the range of 0 to 15 μg of protein. At limiting concentrations of R, the relative activity of terminator codons in stimulating f[^{3}H]-methionine release from ribosomes was as follows: UAA > UAG > UGA. These results confirm the report of Capecchi (*3*) that a release factor is required for UAG-dependent termination of protein synthesis and also show that a release factor is required for UGA- and UAA-dependent termination. Thus, trinucleotides can be used for the purification and characterization of codon-dependent R factor or factors.

It should be possible to determine the translation of terminator trinucleotide codons by assaying either the formation of f[^{3}H]-methionine, the disappearance of f[^{3}H]-Met-tRNA bound to ribosomes, or the disappearance of TCA-precipitable f[^{3}H]-Met-tRNA. A comparison of results obtained by the three assays is shown in Fig. 3. The rate of formation of f[^{3}H]-methionine was approximately the same as the rate of disappearance of ribosomal-bound or TCA-precipitable f[^{3}H]-Met-tRNA. Hence the results obtained by the three assays agree well with one another. Since the appearance of f[^{3}H]-methionine is a more sensitive method of assay than the disappearance of f[^{3}H]-Met-tRNA, f[^{3}H]-methionine formation was routinely used to determine the translation of terminator codons.

Table 4. Possible sets of terminator codons.

Anticodon species	Terminator codon sets	Equivalent bases in codon
1	UAA	
2	UAG	
3	UGA	
4	UAA UGA	A = G 2nd base
5	UAA UAG	A = G 3rd base
6	UAG UGA	Not acceptable
7	UAA UGA UAG	A = G 2nd base or A = G 3rd base but not both at same time

The sensitivity of R to inactivation is shown in Table 3. The R preparations were inactivated by incubation with ribonuclease-free trypsin, *N*-ethylmaleimide, or by incubation at 100°C. However, incubation of R with T1 ribonuclease or diisopropylfluorophosphate had little effect upon R activity. In other experiments not shown here, no inactivation of R was found after incubation with pancreatic ribonuclease A; in addition, R was found to be nondialyzable. These results suggest that at least one protein with a free sulfhydryl group is present in R, which is required for UAA-dependent termination. These findings are consistent with the report of Capecchi (*3*) that R is not inactivated by pancreatic ribonuclease A or by treatment with periodate. The results, however, do not rule out the possibility that RNA component or components may be required in addition to protein.

The mechanism of termination remains unclear. However, the results suggest that terminator trinucleotides attach to ribosomes and are recognized with high specificity by appropriate molecules; and then ribosomal-bound f-Met-tRNA is converted to free formylmethionine. Whether each terminator codon is recognized by the same, or by different molecular species remains to be determined. However, it is clear that UGA, UAA, and UAG are functionally similar since each stimulates formylmethionine release.

As shown in Table 4, seven kinds of codon sets can be constructed with UAA, UAG, and UGA. Nevertheless, only two general kinds of codon recognition mechanisms seem feasible; (i) one "anticodon" responds only to UAA, another to UAG, and a third to UGA; and, (ii) one "anticodon" interacts alternately with A or G in the second or in the third base positions of terminator codons, but not at both positions simultaneously. Thus, a logical mechanism of codon recognition is predicted if one anticodon translates two or three terminator codons. We rule out the possibility that A is equivalent to G simultaneously at the second and third base positions of codons, because molecules translating terminator codons would also respond to the tryptophan codon UGG. Hence the codon set, $\frac{\text{UAG}}{\text{UGA}}$ and seven additional sets of codons (not shown in Table 4) composed of one or two terminator codons and UGG, are excluded. Translation of the three terminator codons by one "anticodon" seems unlikely but cannot be ruled out.

It is clear that the pattern of degeneracy observed with terminator codons differs strikingly from degeneracy paterns found with codons for aminoacyl-tRNA. An equivalence of A and G in the second position of mRNA codons and the recognition only

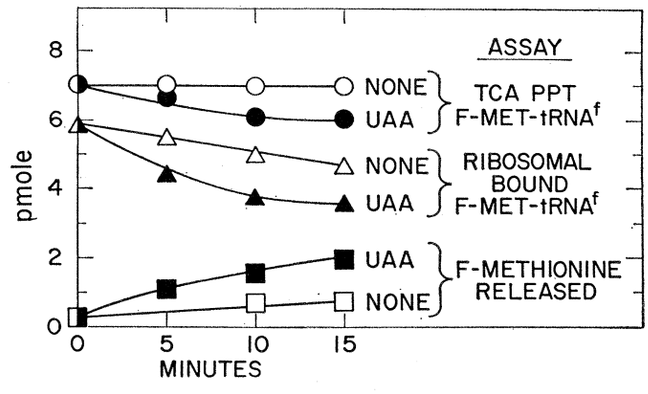

Fig. 3. The effect of UAA upon TCA-precipitable f[^{3}H]-Met-tRNA (3°C), f[^{3}H]-Met-tRNA^f bound to ribosomes, and f[^{3}H]-methionine formation is shown. Each point corresponds to a 0.05 ml reaction containing the components described in the legend accompanying Fig. 1 (including 5.90 pmole of f[^{3}H]-Met-tRNA^f • • • AUG • • • ribosome complex and 11.2 μg of R protein (prep P). Where indicated, f[^{3}H]-Met-tRNA^f was precipitated with 1 ml of 10 percent TCA at 3°C, and the precipitate was washed on a cellulose nitrate filter with 50 ml of 5 percent TCA at 3°C. The assay for ribosomal bound f[^{3}H]-Met-tRNA^f has been described (*20*). The assay for f[^{3}H]-methionine is described in the legend to Fig. 1.

of A in the third position of mRNA codons has not been observed previously. Therefore, terminator codon recognition must differ in some respects from the recognition of other codons by aminoacyl-tRNA (*15*).

Further study is required to identify the kind of molecule that translates terminator-codons and the ribosomal site of translation. A protein apparently is required for termination, but its function has not been defined. It should be possible to clarify the function of R and elucidate the terminal steps of protein synthesis with the experimental approach described.

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Occupancy Principle for Radioactive Tracers in Steady-State Biological Systems

Abstract. *Bergner's theoretical analysis of tracer dynamics has important applications in biology, not only for the measurement of exchangeable mass (as he demonstrated) but also for elicitation of otherwise inaccessible information on total masses of particular elements in body organs or systems—as well as in chemical engineering and geophysics.*

The occupancy principle (which we define) provides a means of determining the total flow of the material under study into a system, and the quantity of that material in any part of the system for which radioactivity can be quantitatively measured.

A steady-state system is one through which there is a continuous flow of material, and in which the quantity of material in any part of the system, and the rate of flow between any two parts, remain constant. Many biological systems behave in ways that closely approximate to this state—for example the utilization of essential dietary constituents by the human body.

Under ordinary conditions, there is no way of distinguishing one part of the entry flow into the system from another; nothing within the system indicates directly to the observer that there is in fact any flow. Such a system can be studied only when a tracer, possessing distinctive characteristics recognizable by the observer, constitutes part of the entry flow.

The occupancy principle states a relation among three parameters; occupancy, capacity, and flow. At some time t after the commencement of administration of the tracer, a defined part of the system contains a fraction $f(t)$ of the total tracer, and this fraction varies with time. The values of $f(t)$ at all times are represented simply by the curve of activity against time for the defined part of the system.

The occupancy Θ for any part of the system is defined as the total integral, with respect to time, of the tracer fraction $f(t)$ that is in that part of the system:

$$\Theta = \int_0^\infty f(t) \cdot dt$$

This is simply the total area under the activity time curve for that part of the system.

The capacity C of any part of the system is the quantity of the material under study (not the tracer) that is in that part at all times; it can be expressed in any terms appropriate to the material. Capacity has the same meaning as "amount of mother substance" or "pool size."

The material enters the system at a constant flow rate F; the entry comes, or can be regarded as coming, entirely from outside the system; therefore a recirculation cannot be part of the entry flow. The occupancy principle states that the ratio of occupancy to capacity is the same for all parts of the system and equals the reciprocal of the entry flow. The principle is valid regardless of the time course of administration of tracer; its proof is straightforward (*1*).

For extension of proof to cover the case in which the tracer is not administered instantaneously, let a fraction K of the tracer be administered at a time t after the measurement of tracer activity. The pattern of flow—that is, the shape of the activity time curve for this fraction—is independent of t. With normalization to $t = 0$, the area under the activity time curve for this fraction is also independent of t; so that the total integrated area under the normalized activity time curves, corresponding to all the fractions of tracer administered at different times t, is independent of t. Therefore the occupancy principle is valid regardless of the time course of the administration of tracer, provided that the total activity of the tracer has been measured at some time before commencement of administration, and that all activities are normalized to that time.

The problem of prolonged tracer administration has been analyzed by Bergner (*2*) for the general case, and the limited case in which only the input and output to the system are accessible has been discussed by Zierler (*3*). Use of the occupancy principle has the effect of transferring the information available from the tracer directly to the material under study. Attention is therefore focused on the real system instead