during photosynthesis by green leaves. The amounts of all FMN-requiring enzymes that cause oxidation and decarboxylation are not known, but other studies (*11*) strongly indicate the importance of glycolic acid oxidase in the respiratory metabolism of leaves.

The importance of this carbon dioxide production to the carbon balance of the plant can be seen if we take carbon dioxide compensation of leaves as about 50 ppm (in many leaves it is higher) and the amount of carbon dioxide in the atmosphere as 300 ppm. Under these conditions carbon dioxide production is responsible for a 17-percent reduction in the net rate of photosynthesis when availability of carbon dioxide is limiting the rate. Corn shoots were the exception to this; carbon dioxide compensation was zero.

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Chain Initiation and Control of Protein Synthesis

Abstract. *Analysis of the enzymatic mechanism of chain extension during protein synthesis and studies with* N-*formylmethionyl-sRNA suggest that chain initiation requires formylation of the amino group of the amino acid destined to start chain growth. The existence of a set of starting triplets coding for a special set of* N-*formylaminoacyl-sRNA's is postulated. These triplets might be ambiguous in the sense that they specify different amino acids, depending on whether they are at the beginning of or within a message. A number of starting triplets and their* NH_2-*terminal amino acids are predicted from previously suggested ambiguities. The biochemical, regulatory, and genetic implications of a formylation step controlling chain initiation are discussed.*

Analyses of the effect of puromycin on the size distribution of polysomes in vivo led me and my associates to postulate that the initiation of a new polypeptide chain upon attachment of a ribosome to the beginning of messenger RNA requires a special mechanism that is separable from the read-out process or chain extension (*1, 2*). This idea has received further support from an examination of the interaction of transfer RNA with ribosomes during chain extension. Available evidence (*2*) is compatible with the scheme of chain extension (Fig. 1). An important aspect of this scheme is the postulate that during the steady-state process of chain extension the ribosomes oscillate between two states, A and B, characterized by the presence of the growing polypeptide chain linked to sRNA (*3*) in either of two sites, α or β (*4*). Thus, with the formation of each peptide bond the nascent chain is transferred to the incoming aminoacyl-sRNA, while the preceding sRNA, now discharged, remains firmly bound to the β-site (Fig. 1, *a* and *d*). In the subsequent step, the GTP-dependent enzyme translocase advances the messenger by one triplet and thereby returns the peptidyl-sRNA to the β-site on the 50S subunit, displacing the discharged sRNA into the loosely binding exit site γ (Fig. 1*b*). The α-, or decoding, site is now vacant until filled with the aminoacyl-sRNA specified by the newly exposed codon (Fig. 1*c*). Our failure to detect aminoacyl-sRNA bound to active ribosomes indicates that this state is very short-lived, evidently because the ribosome-bound peptide synthetase triggers peptide-bond formation at the very instant the correct aminoacyl-sRNA is captured.

If we now try to apply this scheme to the situation at chain initiation, a rather perplexing difficulty arises: what induces the translocase to act whenever the initial aminoacyl-sRNA has been selected by the first triplet in the decoding site? For, as evident from the right hand side of Fig. 1, this constellation differs radically from the situation encountered during chain extension. It would seem to violate all rules of enzyme specificity if an aminoacyl-sRNA with a predominantly charged NH_3^+ group could serve as a substrate for an enzyme that normally recognizes the uncharged amide group of peptidyl-sRNA in this position. A similar situation presents itself with respect to peptide synthetase in the subsequent step. This enzyme is likewise normally confronted with a neutral peptidyl-sRNA rather than with a protonated acyl-sRNA in the condensing site.

The simplest solution to this paradox would be to postulate that chain initiation requires a special derivative of the first amino acid, possibly coded for by a special starting triplet. Furthermore, the postulated derivative should have the charged NH_3^+ group masked to make it look more like peptidyl-sRNA which is what translocase normally recognizes. At the same time it should be rather labile. A derivative that answers this description would be obtained by *N*-formylation. The discovery of *N*-formylmethionyl-sRNA (*5*) lent substance to this hypothesis and suggested that it might function in chain initiation.

After we had started experiments designed to test this hypothesis, further data on *N*-formylmethionyl-sRNA appeared, which strongly indicated its role in chain initiation, although this possibility was not mentioned by Clark and Marcker (*6*). Among their findings the following are of particular relevance to the problem of chain initiation. (i) At least two methionyl-sRNA species exist in *Escherichia coli*, and at least one of them cannot be formylated; (ii) formylation occurs only after methionine has been linked to sRNA; (iii) when a mixture of formylated and nonformylated methionyl-sRNA was offered to a ribosomal incorporation system containing polyUG, methionine, and phenylalanine were incorporated in a ratio of about 1 to 8, and 80 to 90 percent of the TCA-insoluble methionine was recovered as the NH_2-terminal *N*-formyl derivative; (iv) addition of N^{10}-formyltetrahydrofolic acid increased the

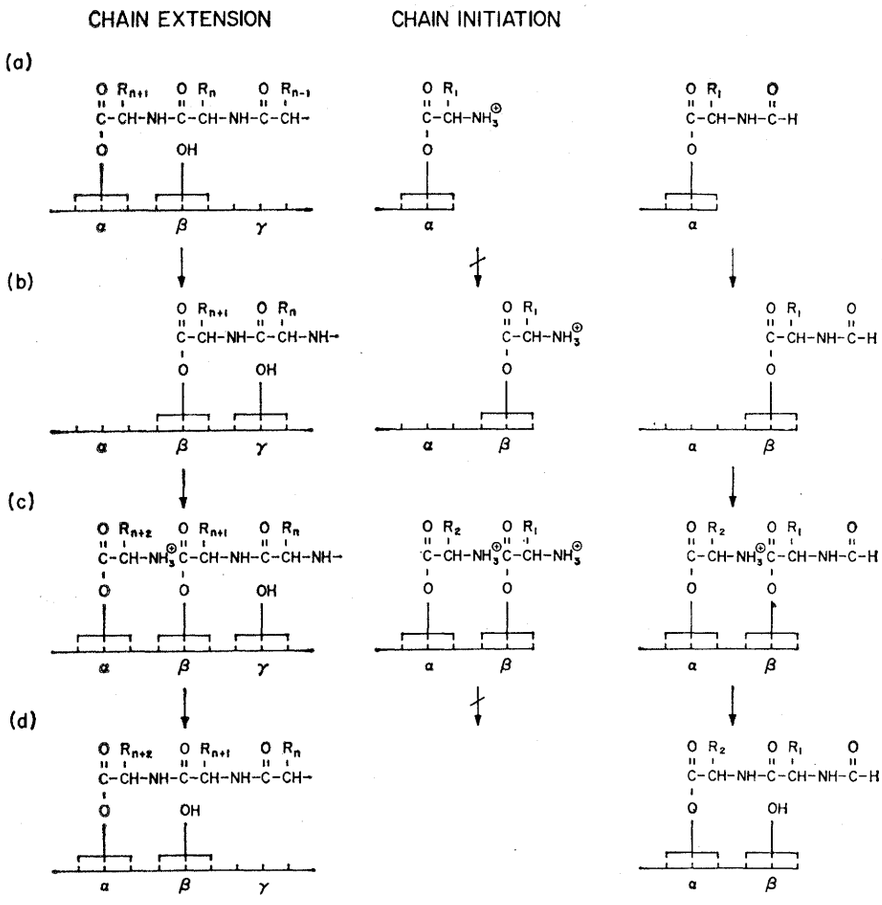

Fig. 1. Model of postulated sequence of events during initiation and extension of polypeptide chain at ribosomal surface. Addition of one amino acid proceeds through stages *a* to *d*, as explained in the text.

incorporation of methionine; and (v) the same extent of specific binding was observed when ribosomes were exposed to either the formylated or nonformylated derivative of methionyl-sRNA in the presence of polyUG or polyAUG, or the trinucleotides UUG or AUG.

These results strongly suggest that only the *N*-formyl derivatives of aminoacyl-sRNA's are capable of chain initiation, even though the coding triplets of the messenger fail to discriminate between the formylated and nonformylated form of a given sRNA. On the other hand, these findings also indicate that a coding triplet may have a different meaning, depending on whether it is at the beginning of or within a message. This apparent contradiction may be resolved if we postulate that the NH_3^+-leucyl-sRNA normally responding to UUG is rejected whenever UUG is in the starting position, because the charged NH_3^+ group cannot bind to the translocase. On the other hand, *N*-formylmethionyl-sRNA will be selected for chain initiation because it is acceptable to the enzyme. Exactly the reverse should hold with respect to the same codon in the nonterminal position: here the *N*-formylated sRNA cannot be used, since the peptide synthetase requires a free NH_3^+-group for peptide-bond formation. An important additional condition is the requirement that the UUG-specific methionyl-sRNA must not occur in nonformylated form; otherwise it would compete with leucine for positions within a chain.

The most startling aspect of these observations is the use of an ambiguous triplet for chain initiation. If the coding ambiguity displayed by UUG is representative of an entire set of starting triplets, we should discover additional coding ambiguities that would allow us to deduce the corresponding set of NH_2-terminal amino acids. Indeed, the data summarized in Tables 1 and 2 reveal a number of such ambiguities. For comparison, I have compiled a list of proteins with known NH_2-terminal amino acids (Table 3). It is immediately obvious that certain amino acids, notably alanine, aspartic and glutamic acids, glycine, serine, and others (class I) are encountered with unusually high frequency in NH_2-terminal position; others (class II), such as proline, cysteine, tryptophane, arginine, and histidine, rarely, if ever. Moreover all of the amino acids coded by the postulated starting triplets fall into the class of amino acids that have been found in NH_2-terminal positions (Table 4), and none of them fall into class II representing nonterminal amino acids. That only about half of the known NH_2-terminal amino acids have been predicted from ambiguity is not surprising, since only somewhat less than half of all 64 triplets have been tested against all 20 amino acids. There is, of course, always the possibility that some or all of the observed ambiguities are artifacts resulting from the special conditions of the particular testing systems in vitro. Some of the ambiguous or starting triplets show degeneracy of a systematic type; they share the last two nucleotides, but differ with respect to the first (Table 2).

The impression that the type of amino acids occurring in NH_2-terminal position is highly restricted (Table 1) is further strengthened by the results of end-group analyses of the total proteins from *E. coli* (*7*). When the two sets of data in Table 4 are compared, the fact that alanine occurs in both sets one out of three times is rather striking. However, three additional amino acids occur with very high frequency among the NH_2-terminal amino acids in the proteins of *E. coli*. Methionine and serine are about as frequent as alanine, threonine about half as frequent. By contrast, in the set corresponding to NH_2-terminal amino acids from pure proteins of all sources, none of these three amino acids is significantly more frequent than the others; on the contrary, methionine and threonine are perhaps less frequent or altogether absent. Even if the discrepancy between the two sets should prove not significant, we would still have to explain the high frequency of NH_2-terminal alanine in both sets and of NH_2-terminal methionine, serine, and threonine in the total proteins of *E. coli*. Any interpretation of these ob-

Table 1. Ambiguous triplets possibly coding for chain initiation. Highly uncertain assignments are in parentheses. The ambiguous codon AAG is from reference (*24*), all others are from reference (*25*).

Codon	Responding amino acid	NH_2-terminal	Nonterminal
UUG	Met, Leu	Met	Leu
UGC	Ala, AsN	Ala, AsN	Cys
UGA	AsN, Glu	AsN, Glu	(Try)
CCA	Pro, Lys	Lys	Pro
CUG	Leu, Met	Met	Leu
CGC	Arg, Ala	Ala	Arg
AAG	Asp, Lys	Asp	Lys
ACA	Thr, Lys	Lys	Thr
AGC	Ala, Ser	Ala (Ser)	Ser or nonsense
AGA	Glu, Arg	Glu (Arg)	Arg or nonsense
GAA	Glu, Lys	Lys	Glu

servations is obviously dependent upon the still unsolved question whether methionine is the only amino acid that can be formylated and hence is the universal chain initiator. If the affirmative view is taken, additional mechanisms must be postulated for the high frequency of the other NH_2-terminal amino acids. For example, whenever NH_2-terminal methionine is followed by either alanine or serine or threonine, or other, the terminal methionine might subsequently be removed, leaving any of these other amino acids in the NH_2-terminal position.

If temporary blocking of the NH_2-terminal amino group by formylation is a general mechanism of chain initiation, subsequent removal of the formyl group must be accounted for. Since the ribosome-bound polypeptide product formed in response to polyUG contained NH_2-terminal methionine in the formylated form, it appears that deformylation occurs late, perhaps after chain release. In some cases the formyl group might be preserved; it is interesting in this connection that gramicidin A contains an *N*-formyl group at the amino terminus (*N*-formyl-L-valine or *N*-formyl-L-isoleucine) (*8*). However, the relevance of this example remains uncertain until it has been firmly established whether or not this polypeptide is synthesized on ribosomes (*9*).

Table 2. Degeneracy of ambiguous codons. The data in Table 1 have been rearranged according to amino acid to show degeneracy of ambiguous codons. Bases that are identical in the last two positions within a set of postulated starting codons are in italics. Also in italics are the same two bases when they occur in the first two positions of the corresponding nonterminal codon.

Amino acid	Codon	Codon
Met	U*UG*, C*UG*	AUG
Ala	UG*C*, CG*C*, AG*C*	G*C*U, G*C*A G*C*C, G*C*G
AsN	UGC	AAU, AAC
Lys	AC*A*, CC*A*, GAA	AAA, AAG
Asp	AAG	GAU, GAC
Ser	AGC	UCU, UCC, UCA, UCG
Glu	AG*A*, UG*A*	G*AA*, G*AG*

The possibility has been raised that in certain cases *N*-acetylation may be used for chain initiation, as suggested by the presence of *N*-terminal acetyl groups in a number of polypeptides and proteins (*10*). A careful examination of several cases, however, indicated that the observed *N*-acetylations take place after the polypeptide chains have been completed (*11*). Whether this applies also to *N*-acetylated virus proteins (as those of TMV and TYMV) is not known.

The postulate, that special starting triplets as well as *N*-acylation of the terminal amino acid are obligatory requirements for chain initiation, seems to be in conflict with the well-known ability of various synthetic polynucleotides (polyU, -A, -C, and others) to initiate chains. This difficulty is easily resolved, however, if we make the plausible assumption that the uncharged amino group of an sRNA-linked amino acid, in contrast to its protonated form, allows binding to the transfer enzymes, though at a reduced rate. Since the *pK* of the amino group of amino acid esters is close to 8 (*12*), almost half of the aminoacyl-sRNA's are in the uncharged form at the high *p*H used in cell-free incorporation systems. Even so, attachment is rather poor and rate-limiting in polyU-dependent polyphenylalanine synthesis (*13*). Under physiological conditions, at a *p*H near 7, the contribution to chain initiation by aminoacyl-sRNA's with uncharged amino groups would be correspondingly smaller.

Another argument, at first sight, against the requirement of special starting triplets for chain initiation, comes from analysis of puromycin action. Puromycin causes release of nascent protein chains from ribosomes and, much less frequently, separation of ribosomes from mRNA. Ribosomes that have lost their nascent chains but remained attached to the messenger are capable of starting new chains (*14*). It is not known whether chain initiation after puromycin action requires *N*-formylated aminoacyl-sRNA. Puromycin appears to cause tracking errors and slipping (*2, 15*) which might lead to out-of-phase reading of the messenger. As a consequence, both "starting" and "release" triplets would be encountered with a certain frequency within a message. Chain initiation could then proceed in the usual manner.

The biochemical, regulatory, and genetic implications of a formylation

Table 3. NH_2-terminal amino acids of known proteins.

Protein	Reference
Alanine	
Amylase (hog pancreas)	(*26*)
α-Amylase (*Aspergillus oryzae*)	(*27*)
Ferredoxin (*Clostridium pasteurianum*)	(*28*)
Cytochrome c_3 (*Desulfovibrio desulfuricans*)	(*29*)
Cytochromoid (*Rhodospirillum rubrum*)	(*29*)
Cytochromoid (*Chromatium*)	(*29*)
Prothrombin	(*30*)
Fibrinogen	(*30*)
Cornalbumin	(*30*)
Transferrin	(*30*)
7S γ-Globulin (hog)	(*30*)
Glutamic acid or glutamine and aspartic acid or asparagine	
Cytochrome c_2 (*R. rubrum*)	(*29*)
Cytochrome *c* (*Chromatium*)	(*29*)
Fibrinogen	(*30*)
Serumalbumin (mammal, bird)	(*30*)
7S γ-Globulin (man, horse, hog)	(*30*)
Enterotoxin B (*Staphylococcus*)	(*31*)
Carboxypeptidase	(*32*)
Glycine	
Amylase (hog pancreas)	(*26*)
N-*Acetylglycine*	
Cytochrome *c* (man, horse, hog)	(*33*)
Cytochrome *c* 552 (*Euglena*)	(*29*)
Hemoglobin F_1	(*34*)
Valine	
Hemoglobin α, β	(*35*)
Trypsinogen	(*32*)
7S γ-Globulin (ox)	(*30*)
Isoleucine and leucine	
Papain	(*36*)
Pepsinogen	(*32*)
Fetuin	(*30*)
Serine	
7S γ-Globulin (human, horse, ox)	(*30*)
N-*Acetylserine*	
Melanocyte stimulating hormone (α MSH)	(*37*)
TMV coat protein	(*38*)
Lysine	
Lysozyme (egg albumin)	(*39*)
Ribonuclease A (ox pancreas)	(*40*)
Methionine	
Tryptophan synthetase A-protein (*E. coli*)	(*41*)
N-*Acetylmethionine*	
TYMV coat protein	(*42*)
Phenylalanine	
Amylase (hog pancreas)	(*26*)
α-Amylase (*Bacillus stearothermophilus*)	(*43*)
Tyrosine	
Fibrinogen	(*30*)

Table 4. Occurrence of amino acids in NH_2-terminal position. None fall into class II (proline, cysteine, tryptophane, arginine, histidine). The column headed "predicted" means predicted from ambiguous triplets.

Amino acid	NH_2-terminal		Relative frequency (%)* in proteins	
	Found	Predicted	*a* Pure (all sources)†	*b* Total (*E. coli*)‡
Class I				
Methionine	+	+	5	30
Alanine	+	+	29	24
Serine	+	+	8	20
Threonine	+§	—	0	12
Aspartic acid, glutamic acid, and amides	+	+	18	7
Glycine	+	—	10	3
Isoleucine-leucine	+	—	8	2
Lysine	+	+	5	1
Valine	+	—	8	1
Class I, but not present in E. coli				
Phenylalanine	+	—	5	0
Tyrosine	+	—	3	0

* The relative frequency is not strictly comparable in *a* and *b*, since it is defined differently; in *a*, frequency of any particular NH_2-terminal amino acid is percentage of total number of all proteins listed in Table 2; in *b*, frequency is in moles per 100 moles of end groups found. † Data from Table 2. ‡ Data from (7). § Not present in compilation of pure proteins.

step controlling chain initiation are sufficiently important to merit serious consideration. Biochemically, such a mechanism would place nucleic acid and protein synthesis under the common control of the supply of active one-carbon (C_1) fragments, such as methyl, methylene, carboxyl, and others. In particular, protein synthesis would be critically dependent upon cofactors involved in formyl transfer, such as pyridoxal phosphate and tetrahydrofolic acid. Conversely, antagonists of these cofactors, like deoxypyridoxine and 4-amino-N^{10}-methylpteroylglutamic acid (methotrexate), are expected to inhibit protein synthesis by blocking chain initiation. There are many indications that point in this direction. Thus, the formylation hypothesis would explain the hitherto obscure inhibitory action of deoxypyridoxine on protein synthesis. Particularly persuasive are studies of hemoglobin synthesis in reticulocytes. These cells contain high levels of the formate-activating enzyme that generates N^{10}-formyltetrahydrofolate (*16*). It is perhaps significant that, of all cell-free systems tested so far, only the reticulocyte system shows significant chain initiation in vitro. We are now testing whether addition of a formyl generating system will repair the deficient chain initiation activity observed in other cell-free systems (*2*).

The formylation hypothesis also predicts that blocking of chain initiation by inhibition of formylation in vivo should lead to polysome breakdown. Indeed, the polysome breakdown observed in the liver of rats treated with ethionine (*1*) may have been caused to a larger extent by the blocking of chain initiation due to shortage of C_1 fragments than by inhibition of mRNA synthesis. The extremely rapid reassembling of single ribosomes into polysomes after administration of adenine and methionine would be compatible with this view, since it would only require the reattachment of ribosomes to a store of mRNA already present.

An intriguing aspect of the regulation of chain initiation by formylation is the specificity of the transformylation enzyme with respect to the aminoacyl-sRNA responding to the starting triplet. The postulated transformylases share with the corresponding activating enzymes the ability to recognize simultaneously a certain sRNA and its cognate amino acid. It would therefore be economical if both functions, activation (aminoacylation) and *N*-formylation, were carried out by the same enzyme. Such an arrangement, by preventing the coexistence of the formylated and nonformylated forms, would automatically ensure unambiguous translation.

The formylation hypothesis might also offer a clue to the mechanisms responsible for such dramatic metabolic shifts as occur in virus infection, tissue regeneration (liver), and antibody formation. It has long been a puzzle how an infecting RNA strand of a virus succeeds in taking over the ribosomes for its own translation by displacement of the host messenger. The rapid breakdown of the host polysomes after HeLa cells are infected with poliovirus (*17*) would be expected if the virus interfered with the *N*-formylation of the host's aminoacyl-sRNA's corresponding to the starting triplets. Conceivably, the virus genome codes for an inhibitor of the transformylase and, in the case of TMV and TYMV, for a transacetylase that specifically acetylates one particular aminoacyl-sRNA corresponding to the starting triplets of the viral message. In this way, the preferential reading of the viral message would be ensured. In the case of L-cells infected with Mengo virus, there is evidence that the inhibition of synthesis of host protein is caused by protein or proteins synthesized under the control of the viral RNA (*18*). The need for specific acylated sRNA's capable of recognizing the starting triplets of viral RNA might also explain the host specificity of RNA viruses.

The consequences of the hypothesis of formylation for the interpretation of genetic data have been discussed (*19*). An interesting case is virus RNA, since one would expect that mutations in the starting triplet are either lethal or, in the case of transitions within a set of degenerate codons, undetectable. Indeed, no amino acid replacements have so far been detected in the NH_2-terminal position of TMV coat protein (*20*). While it is clear that a mutational change in the starting triplet would usually render an RNA message untranslatable, it is questionable whether such messages would be at all produced. Synthesis of messenger would be inhibited if, as we have proposed earlier (*21*), ribosomes initiated the formation of polysomes by attaching themselves to the free end of the nascent messenger and peeling it off the DNA template. In addition, since viral RNA replicases are specific for a given RNA (*22*), that is, perhaps capable of recognizing a characteristic starting triplet or sequence, we must now consider the possibility that the initiation of mRNA transcription is itself dependent upon the postulated starting triplets. Hence, mutations of the DNA starting triplets might directly prevent the synthesis of the cognate messengers (*19, 23*).

Note added in proof. The idea that methionine is a universal chain initiator is supported by the isolation of *N*-formylmethionyl-sRNA from rabbit reticulocytes. However, no formylated methionine was detected in the nascent protein (H. Küntzel and H. Noll, in preparation).

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Energy Balancing in Nitella Cells Treated with Dinitrophenol

Abstract. *The toxic action of 2,4-dinitrophenol on the large cells of the alga,* Nitella clavata, *was evaluated, with the rate of protoplasmic streaming and the survival time at three light intensities used as criteria. At a sufficiently high intensity the cells survived several weeks, an indication that the energy-uncoupling action of dinitrophenol could be counterbalanced to some extent by an increased energy input. In the treated cells chloroplasts moved from the outer gel-type cytoplasm into the inner, streaming cytoplasm.*

The rate of protoplasmic streaming in the large internodal cells of *Nitella clavata* is dependent on the cellular energy supply (light intensity) only at low levels of illumination (*1*). Moreover, the quantitative relation between streaming rate and light intensity has been demonstrable only in cells kept under constant conditions for several weeks. It can be assumed that energy storage products in the cell had to be depleted before the cell became a stationary system, at least with regard to the flow of energy.

We now report the opposing effects of 2,4-dinitrophenol (DNP) and light on the energy balance of *Nitella*. A light intensity of sufficient magnitude serves to counterbalance the toxic action of $2 \times 10^{-5} M$ dinitrophenol at *p*H 6.7 (Fig. 1). All cells at the two lower light intensities died. At the highest light intensity (2700 erg cm^{-2} sec^{-1}, corresponding to about 540 lux, from incandescent lamps) an apparently stationary state is attained within 2 days, with the streaming rate reduced to about 70 percent of the control value.

The quantitative relationship for untreated cells (*2*) is as follows: at the "bare-survival" light intensity of 120 erg cm^{-2} sec^{-1} the streaming rate is 60 percent of the maximum (saturation value); the streaming rate increases with increasing light intensity until the maximum is attained at 600 erg cm^{-2} sec^{-1}, remaining at this level for higher intensities. If the relative streaming rate of DNP-treated cells is a valid index of the cellular energy supply, then the cells at 2700 erg cm^{-2} sec^{-1} are comparable to untreated cells slightly above

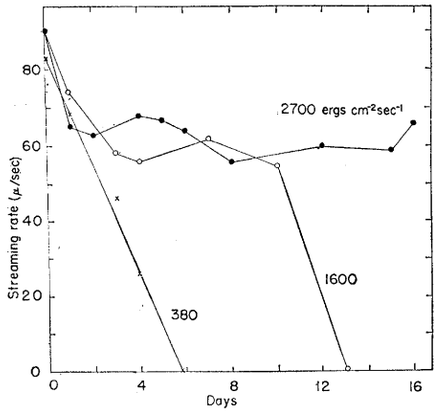

Fig. 1. The influence of light intensity on the rate of protoplasmic streaming and survival time of typical cells of *Nitella clavata* in a solution (*7*) containing $2 \times 10^{-5} M$ dinitrophenol, *p*H 6.7. Cells having no streaming are dead. The mean streaming rate for control cells at the two higher light intensities was 89 ± 3 μ/sec over the 16-day period. Temperature, 22°C.