A Study of Transfer Ribonucleic Acid in *Neurospora*. II. Failure to Detect Transfer Ribonucleic Acid Alterations in Tyrosinase-Derepressed Cultures*

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ABSTRACT: We have compared the chromatographic profile on methylated albumin kieselguhr columns of aminoacyl transfer ribonucleic acids from vegetative cultures of *Neurospora crassa* with those of cultures which were derepressed for tyrosinase by ethionine. No qualitative transfer ribonucleic acid alterations were observed; the same number of components for each amino acid was found in cultures of both developmental states and they had the same chromatographic mobilities. However, quantitative changes of specific acceptor activity were observed for several amino acids. The time course of the pattern of quantitative alteration suggests that the observed changes result from partial ribonuclease digestion of the transfer ribonucleic acid complement.

We believe that the appearance of ribonuclease activity is related to this developmental transition in *Neurospora* but that the observed quantitative transfer ribonucleic acid alterations are not directly involved in the regulation of the system.

Alterations in the chromatographic profile of aminoacyl-tRNA accompanying certain physiological or developmental changes have been observed recently in several organisms, ranging from bacteria to mammals (Baliga *et al.*, 1968; Doi *et al.*, 1966; Holland *et al.*, 1967; Kano-Sueoka and Sueoka, 1966; Kwan *et al.*, 1968; Lazzarini, 1966; Lee and Ingram, 1967; Subak-Sharpe and Hay, 1965; Taylor *et al.*, 1967; Waters and Novelli, 1967; Wevers *et al.*, 1966; Yang and Comb, 1968). The usual technique used to make such observations is cochromatography on MAK[1] columns of a ^{14}C-labeled amino acid attached to tRNA taken from one physiological or developmental state of an organism and tRNA taken from some other state attached to the same amino acid labeled with tritium. In the most intensively investigated case, Kano-Sueoka and Sueoka (1966) found that the chromatographic pattern of *Escherichia coli* tRNA specific for 1 of 17 amino acids tested, leucine, is altered after T_2 infection. In the other systems investigated the aminoacyl-tRNA affected is not necessarily leucyl-tRNA. In the "adaptor-modification" hypothesis Sueoka and Kano-Sueoka (1964) proposed that tRNA alterations regulate major metabolic transitions at the translational level.

We have investigated whether tRNA alterations are involved in the differentiation of the eukaryote, *Neurospora crassa*. The developmental phenomenon to be examined was the response of vegetative mycelium to "hard times," *i.e.*, starvation in buffer, addition of amino acid analogs, or inhibition of protein synthesis with cycloheximide. Starvation on solid medium results in sexual differentiation, the formation of protoperithecia (N. H. Horowitz and M. Fling, 1966, unpublished data). Protoperithecia do not form in liquid culture, and biochemical operations using cultures growing on solid medium are not convenient. However, since certain of the enzymes characteristic of sexually differentiated cultures, *e.g.*, tyrosinase (Hirsch, 1954), are made in liquid culture in response to hard times (Horowitz *et al.*, 1961), it is presumed that the same mechanisms are operating but that the entire process cannot be completed in liquid culture. This view is supported by the fact that two mutants *ty*-1 and *ty*-2 which in liquid culture do not respond to starvation by synthesizing tyrosinase, when tested on solid media are female sterile; they cannot form protoperithecia (Horowitz *et al.*, 1961).

After initially determining the conditions required for the complete charging of the 20 amino acids of protein to *Neurospora* tRNA (Shearn and Horowitz, 1969), chromatographic comparisons were made between aminoacyl-tRNA from vegetative, control mycelium and aminoacyl-tRNA from cultures which were stimulated to synthesize tyrosinase by treatment with ethionine, a methionine analog which is incorporated into *Neurospora* protein (Kappy and Metzenberg, 1965).

Alterations in the chromatographic profile of tRNA of the type reported for other systems, *i.e.*, changes in the number or mobility of components, were not found

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[1] Abbreviations used in this paper: NADase, nicotinamide-adenine dinucleotide glycohydrase; all other abbreviations are identified in preceding paper.

in this system for any of the 20 amino acids. However, significant loss of total tRNA was observed following ethionine treatment as were changes in the specific acceptor activity for several amino acids. The decline of total tRNA is correlated with a decrease in the rate of tRNA synthesis and the abundance in differentiated cultures of ribonuclease activity. The observed quantitative tRNA changes are probably not directly involved in regulation.

Experimental Procedure

Materials and Methods. Wild-type strain 69-1113a of *N. crassa*, which was used as the source for sRNA, produces high levels of tyrosinase (Fling *et al.*, 1963). The aminoacyl-tRNA synthetase preparation and the chemicals used in this work are the same as described in the previous paper (Shearn and Horowitz, 1969).

The mycelium was cultured according to the short-term procedure developed by Pall (1967). Flasks containing 48-hr mycelium, grown in *subminimal* medium (a 1:2 dilution of medium N (Vogel, 1956) containing 0.5% (w/v) sucrose), are placed on a reciprocal shaker (60–70 excursions/min); after 24 hr a sterile solution of DL-ethionine is added to the medium to a final concentration of 0.1 mg/ml and the shaking is continued. The mycelium is harvested and the tRNA is extracted as described in the preceding paper (Shearn and Horowitz, 1969).

Crude extracts of individual mycelial pads in 0.1 M sodium phosphate buffer (pH 6.0) are used for the assay of tyrosinase (Horowitz *et al.*, 1960) and total soluble protein (Gornall *et al.*, 1949). Aminoacyl-tRNA was prepared and assayed as described in the preceding paper (Shearn and Horowitz, 1969).

Chromatography of aminoacyl-tRNA on MAK, described first by Sueoka and Yamane (1962), was performed on columns prepared by a modification of the technique of Mandel and Hershey (1960). Serum albumin is methylated for 5 days at 35° instead of 3 days a room temperature and is stored as a dried powder over KOH in a dessicator. The column is prepared in a 22-mm diameter chromatography tube. To prepare methylated albumin-coated kieselguhr, 16 g of kieselguhr is suspended in 80 ml of starting column buffer (0.05 M sodium phosphate, pH 6.3, and 0.2 or 0.4 M NaCl). To the 80-ml suspension, 4.5 ml of a 1% solution (in water) of methylated albumin is added slowly with stirring. Another suspension, using 1 g of kieselguhr and 10 ml of buffer, is used for the top layer of the column. The MAK is poured into the tube with a wide-bore pipet and packed down with nitrogen pressure at 3 psi. This process is continued until all of the MAK is poured. The 10-ml kieselguhr suspension is poured as an upper protective layer. The succeeding steps including washing, sample application, and elution are all performed with an applied N_2 pressure of 3 psi. The tRNA is eluted with a linear salt gradient from 0.2 or 0.4 to 1.0 M NaCl. The optical density at 265 mμ of the effluent from the column was continuously monitored with a Gilson ultraviolet absorption meter and recorded on a Texas Instruments rectilinear recorder. Fractions (2 ml) are collected at the rate of 30 fractions/hr. The entire chromatography procedure is performed at 18°.

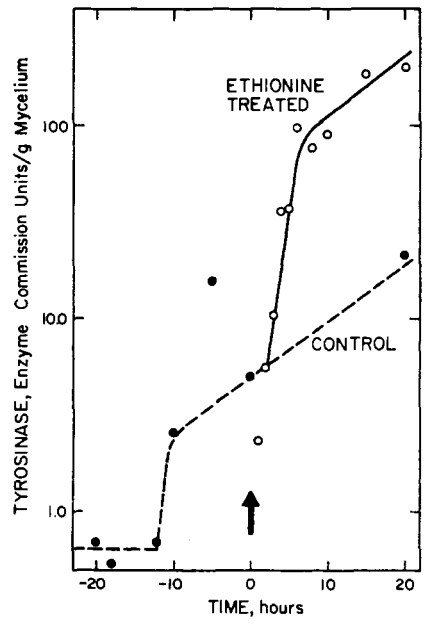
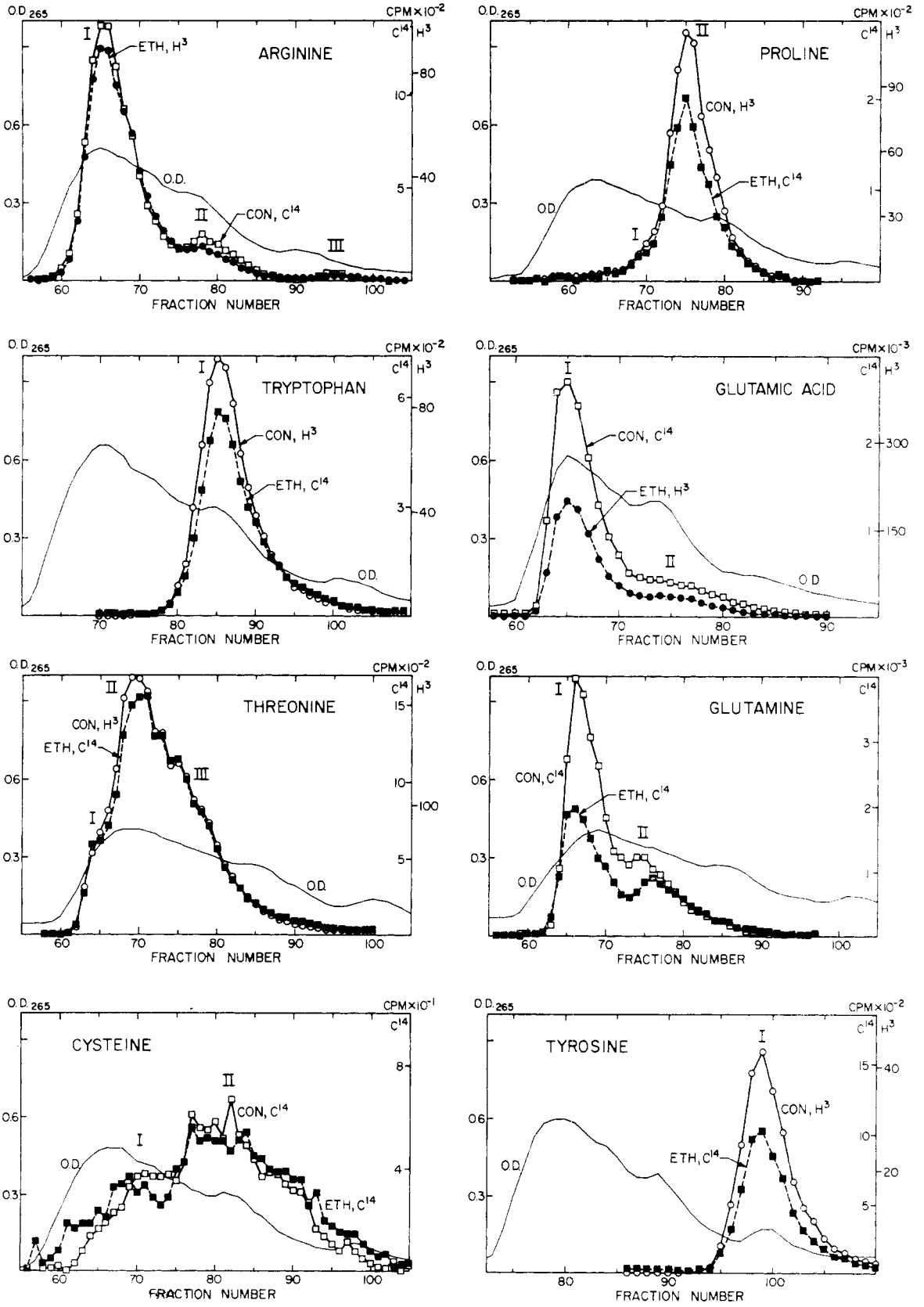


FIGURE 1: Time course of tyrosinase synthesis. The time scale of the ordinate is relative to the time of ethionine addition (arrow). At zero time, according to the figure, a culture is 72-hr old. The first 48 hr are stationary, followed by reciprocal shaking. The tyrosinase values at each point, indicated in Enzyme Commission Units per gram of mycelium (wet weight), are averages of several cultures from different experiments and are plotted on a logarithmic scale. The dashed line and solid circles represent control cultures, never exposed to ethionine. The solid line and open circles represent ethionine-treated cultures.

All radioactivity measurements were made with a Beckman liquid scintillation counter, either Model CPM 200 or LS 200 B. Filter paper or glass fiber disks were counted in 5 ml of solution A (0.01% (w/v) 1,4-bis[2-(4-methyl-5-phenyloxazolyl] and 0.4% 2,5-diphenyloxazole (w/v) in toluene). Doubly labeled (^{14}C and ^{3}H) samples from MAK chromatography were counted directly in a mixture of solution A and Triton X-100, a nonionic detergent (Patterson and Greene, 1965). Each 2-ml fraction was transferred to a vial containing 15 ml of a 2:1 mixture of solution A and Triton X-100 and 1 ml of distilled water. The aqueous phase enters a stable solution after vigorous shaking with a Vortex mixer, and stays in solution as long as the temperature remains between 20 and 30°. There are two main advantages to this mixture: (1) high counting efficiency and (2) ease of sample preparation. It is possible to count samples directly with this mixture because the increasing concentration of salt throughout the gradient does not affect the extent of quenching. The actual proportions of the mixture were dictated by the large sample size and the high ionic strength.

In order to minimize problems arising from differential counting of doubly labeled samples, each chromatographic comparison was made twice, on the same day, each time with a given isotope in the opposite sample. For example, in a chromatographic comparison of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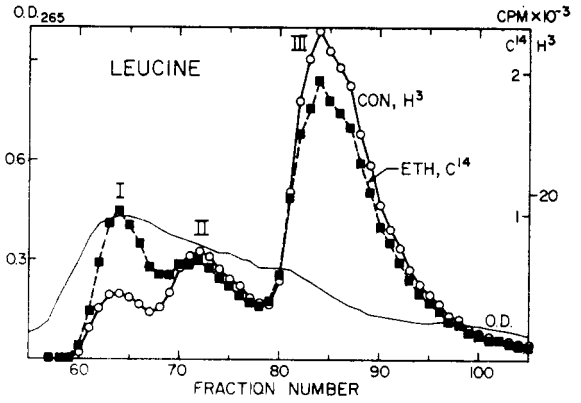
CON, C14
O.D.
I
II
III
PROLINE
CON, H3
ETH, C14
TRYPTOPHAN
GLUTAMIC ACID
THREONINE
GLUTAMINE
CYSTEINE
TYROSINE
O.D.265
CPM×10-2
CPM×10-3
CPM×10-1
C14
H3
FRACTION NUMBER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III
II
I
CON, H3
ETH, C14
O.D.
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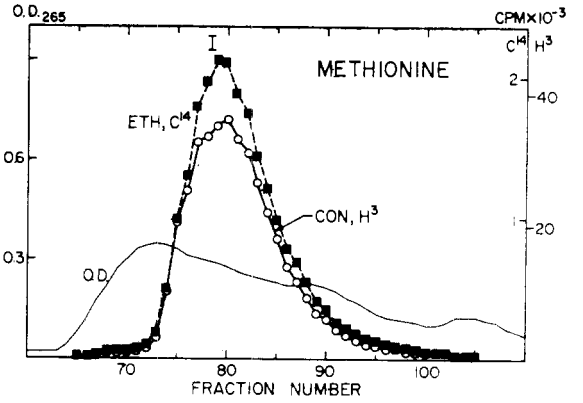
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CON, H3
O.D.
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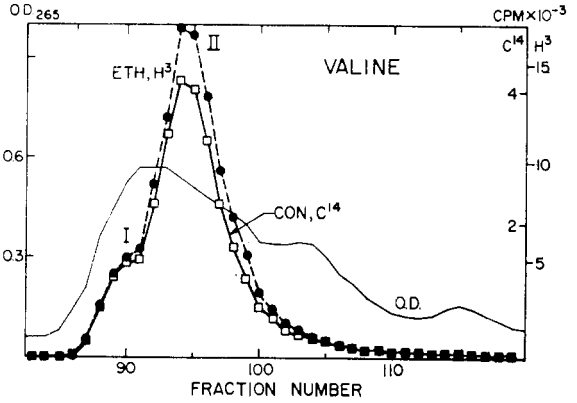
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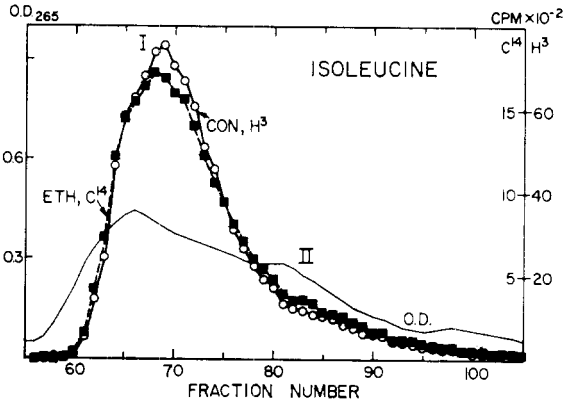
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II
CON, H3
ETH, C14
O.D.
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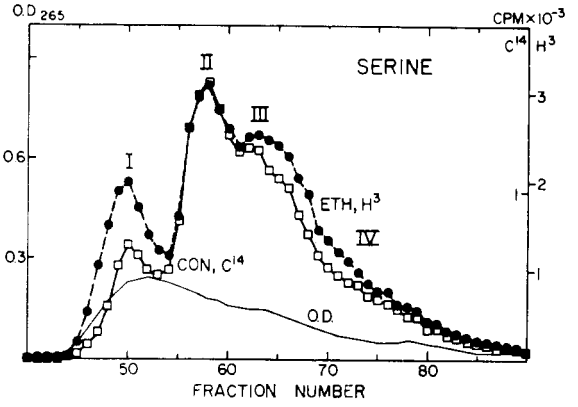
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IV
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CON, C14
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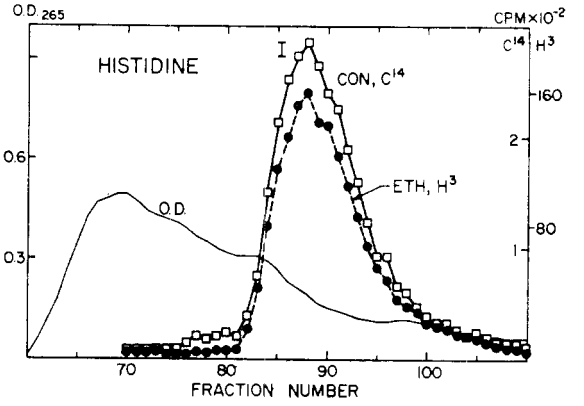
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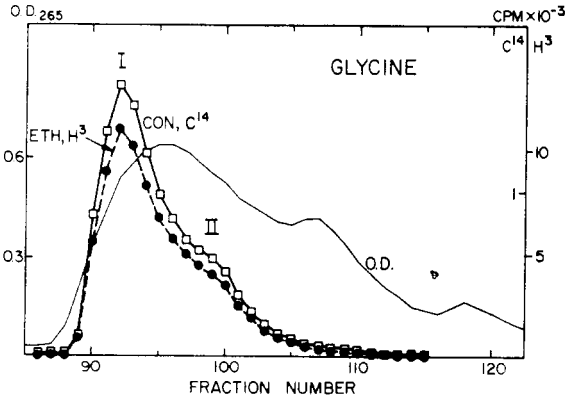
ETH, H3
O.D.
FRACTION NUMBER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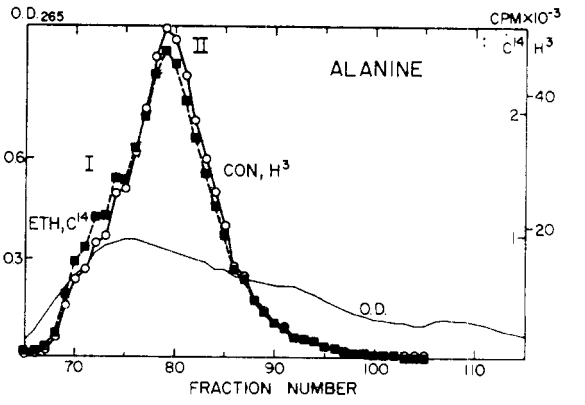
ETH, H3
CON, C14
II
O.D.
FRACTION NUMBER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II
I
CON, H3
ETH, C14
O.D.
FRACTION NUMBER

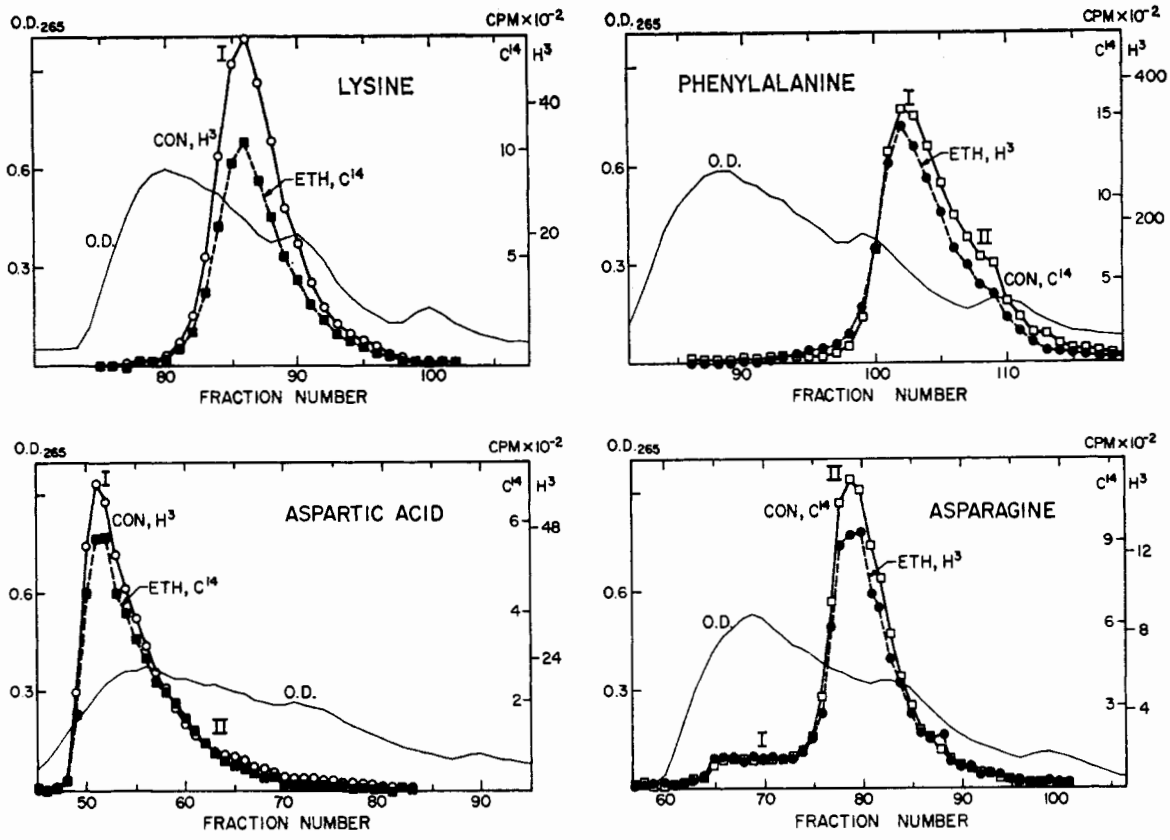

FIGURE 2: Cochromatography of aminoacyl-tRNA from vegetative, control, and ethionine-treated cultures. For 18 of the 20 amino acids, tRNA extracted from one culture was charged with [^{14}C]amino acid and cochromatographed with tRNA extracted from the other kind of culture, charged with the same amino acid labeled with tritium. Tritium-labeling is not available for the other two amino acids, cysteine and glutamine. For these, two columns were run for each comparison using [^{3}H]glycyl-tRNA as a reference marker. Thin line: OD_{265}; heavy solid line and open symbol: tRNA from vegetative control culture (Con); dashed line and filled symbol: tRNA from ethionine-treated culture (Eth); squares: [^{14}C]amino acid; circles: [^{3}H]amino acid. Roman numerals identify each peak and shoulder. See preceding pages for other parts of this figure.

$tRNA_{Leu}$ extracted from vegetative control (Con) and ethionine-treated (Eth) cultures one column would compare [^{14}C]leucyl-$tRNA_{Con}$ with [^{3}H]leucyl-$tRNA_{Eth}$ and the other [^{3}H]leucyl-$tRNA_{Con}$ with [^{14}C]leucyl-$tRNA_{Eth}$. By using the same amount of unfractionated tRNA from each culture on each column, it is possible to calculate a plotting ratio of ^{3}H counts to ^{14}C counts, without requiring an independent determination of the counting efficiency or specific activity of isotope. This enables semiquantitative comparisons to be made by simple inspection of the chromatographic profiles. The plotting ratio is a number which relates ^{3}H counts per minute per micromicromole to ^{14}C counts per minute per micromicromole and is calculated in the following way. Let H = ^{3}H cpm/$\mu\mu$mole and C = ^{14}C cpm/$\mu\mu$mole, where a, b, x, and y are sums of counts per minute recovered from columns.

$$a = \Sigma[^{14}\text{C}]\text{tRNA}_{\text{Con}} \text{ and } b = \Sigma[^{3}\text{H}]\text{tRNA}_{\text{Eth}}$$

$$x = \Sigma[^{3}\text{H}]\text{tRNA}_{\text{Con}} \text{ and } y = \Sigma[^{14}\text{C}]\text{tRNA}_{\text{Eth}}$$

The amount of unfractionated tRNA of both types applied to each column is equal, thus

$$OD_a = OD_b \text{ and } OD_x = OD_y$$

Since specific acceptor activity does not depend upon the isotope used to measure it

$$\frac{a}{OD_a}\frac{1}{C} = \frac{x}{OD_x}\frac{1}{H} \text{ and } \frac{b}{OD_b}\frac{1}{H} = \frac{y}{OD_y}\frac{1}{C}$$

rearranging terms

$$\frac{aH}{Cx} = \frac{OD_a}{OD_x} \text{ which equals } \frac{OD_b}{OD_y} = \frac{bC}{Hy}$$

then

$$\frac{H^2}{C^2} = \frac{bx}{ya} \text{ and } \frac{H}{C} = \left(\frac{bx}{ya}\right)^{1/2}$$

All chromatographic profiles are presented using this relationship except those for glutaminyl-tRNA and cysteinyl-tRNA. For these amino acids tritium labeling was not available.

Results

Preliminary Experiments. After 60-hr growth (48 stationary and 12 shaking) in subminimal medium,

strain 69-1113a begins to synthesize a small amount of tyrosinase. The rate of synthesis quickly declines, so that for the next 24 hr there is only a slow accumulation of enzyme (Figure 1). If ethionine is added at 72 hr after inoculation (indicated by the arrow in Figure 1 and defined here as zero time), then after a 2-hr lag an additional period of rapid synthesis occurs. If no ethionine is added at zero time then 20 hr later there is an accumulation of 21.5 units/g wet weight of mycelium; if ethionine is added at zero time the level after 20 hr is increased to 201.5 units/g. In many strains of *Neurospora* no enzyme appears without the addition of inhibitor; however strain 69-1113a was selected as a high producer of tyrosinase by virtue of its sensitivity to starvation (Fling *et al.*, 1963). The important point is that the addition of the amino acid analog stimulates a rapid net synthesis of tyrosinase. Similar conditions have been shown to also stimulate the synthesis of at least two other enzymes: L-amino acid oxidase (Horowitz, 1965) and NADase (Urey, 1966).

During the period of maximal rate of tyrosinase synthesis (5 hr after addition of ethionine), the rate of general protein synthesis as measured by the incorporation of tritiated phenylalanine into hot trichloroacetic acid insoluble material decreases slightly, but the total soluble protein declines sharply for 10 hr and then levels off. The decline in the rate of protein synthesis is not nearly enough to account for this change in protein content.

Ethionine treatment of mycelium also causes a severe loss of total RNA relative to 0-hr control cultures. This includes a rapid loss of sRNA. Within 5 hr 40% of the sRNA has been lost; this loss increases to 75% by 20 hr. The rate of incorporation of tritiated guanosine into sRNA after 5 hr decreases to roughly 30% of the pretreatment rate. The decreased incorporation may reflect an increase in precursor pool size as well as a decrease in the rate of RNA synthesis. The chromatographic profile of pulse-labeled sRNA, however, remains quite similar.

The extent of methylation of sRNA *in vivo* decreases, but to a lesser extent than does sRNA synthesis. The specificity of methylation as revealed by cochromatography on MAK columns of this *in vivo* methylated sRNA does not significantly change during derepression. No significant ethylation of sRNA occurs in this system. As mentioned in the preceding paper (Shearn and Horowitz, 1969) considerable ribonuclease activity is apparent in the ethionine-derepressed cultures.

Chromatography of Aminoacyl-tRNA. If tRNA modifications were involved in the regulation of the ethionine-mediated metabolic shift described above, then alterations in the chromatographic profile of the tRNA should be observed. This prediction was tested by comparing, on MAK columns, the chromatographic profiles of the aminoacyl-tRNA from vegetative control mycelium (−20 hr) with that of ethionine-treated cultures (+5 hr) which produce tyrosinase at a rapid rate. Figure 2 contains representative profiles of each aminoacyl-tRNA comparison.

These profiles provide data on: (1) the amount of tRNA specific for each amino acid; (2) the number of separable components that compose this amount; and (3) the relative proportion of each component. Finally, they indicate whether any of these parameters are changed in the tRNA extracted from ethionine-treated cultures. Figure 3 summarizes diagrammatically the MAK chromatographic mobilities of the 40 *Neurospora* aminoacyl-tRNAs we have found.

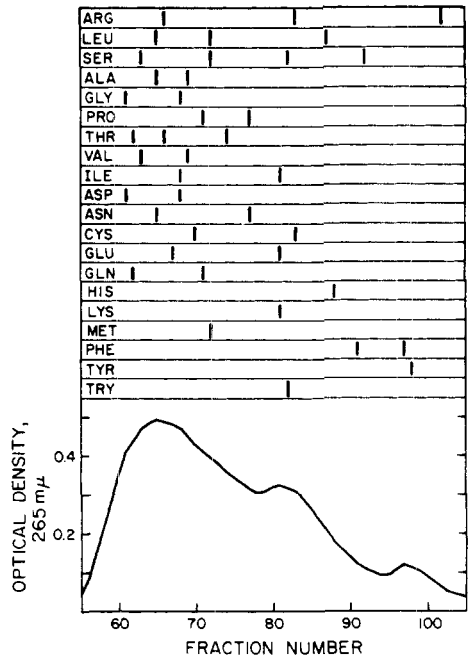

FIGURE 3: Summary of the elution profiles of the 40 aminoacyl-tRNAs in *Neurospora*. The mobility of each tRNA component on MAK for the 20 amino acids of protein is diagrammatically indicated relative to the optical density profile.

No qualitative changes in profile are observed; for each amino acid the same number of components are found in both types of culture, and they have identical chromatographic mobilities. However, significant *quantitative* differences for 10 aminoacyl-tRNAs do exist between the different cultures.

After ethionine treatment, the amount of serine, valine, and methionine tRNA appears to increase, and that for glutamic acid, glutamine, histidine, lysine, proline, tryptophan, and tyrosine decreases.

The relative proportions of the components for 14 of the 15 amino acids with multiple aminoacyl-tRNA components remain the same. The sole exception, glutaminyl-tRNA, seems to lose some of component I but none of component II (Table I). Thus the relative proportion in this case changes considerably.

The observation of a relative increase in the proportion of component I for leucyl-tRNA (Figure 2) is an artifact. It is characteristic, not of the source of tRNA, but of the ^{14}C label. Presumably, the [^{14}C]leucine, but not the [^{3}H]leucine was slightly contaminated with some other amino acid.

The chromatographic differences in the proportions of the four seryl-tRNA components (Figure 2) are not reproducible. Each of seven different chromatographic profiles of seryl-tRNA show unique proportions of the

TABLE I: Change in Proportions of Glutamine tRNA Components.[a]

Sample	Distribution of Components					
	Percentage			$\mu\mu$moles/OD_{260}[b]		
	I	II	Total	I	II	Total
Control	72	28	100	47.8	18.6	66.4
Ethionine treated	60	40	100	26.3	17.6	43.9
Net change				−21.5	−1.0	−22.5

[a] The glutamine specific acceptor activity of tRNA from control cultures, determined by direct assay, is divided between the two components, according to chromatographic evidence. The ethionine-treated culture yielded tRNA with 66.1% as much activity for glutamine and this is also apportioned in the table according to chromatographic evidence. [b] Micromicromoles of glutaminyl-tRNA per OD_{260} of unfractionated sRNA applied to the column.

four components. This result can be explained by postulating either that in each case the seryl-tRNA chromatographed was not completely charged (Shearn and Horowitz, 1969) or that interconversion of the four components occurs. Gartland and Sueoka (1966) have observed interconversion of tryptophanyl-tRNA components in *E. coli*.

Time Course of Quantitative Changes in Acceptor Activity. In order to explore further the quantitative differences indicated by the chromatographic data, tRNA was extracted from five cultures grown for various lengths of time with no ethionine added and seven cultures after varying lengths of ethionine treatment; the tRNA was assayed by a filter paper disk method (Chambers, 1966) for specific acceptor activity (total micromicromoles of amino acid attached per OD_{260} unit of unfractionated tRNA). Each of the 12 tRNA samples was assayed individually for each of the 20 amino acids.

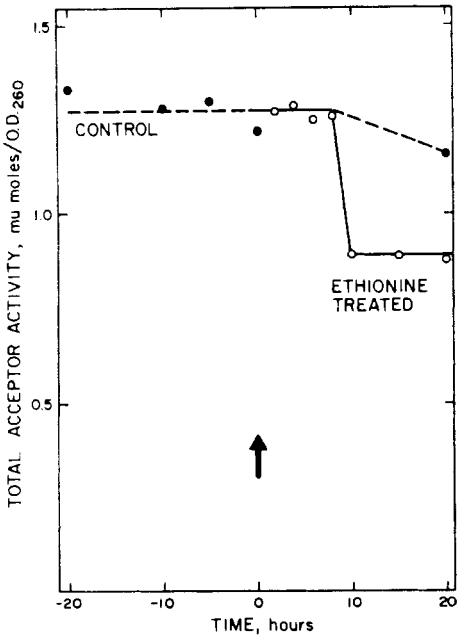

FIGURE 4: Change in total acceptor activity as a function of length of treatment. Solid circles: untreated control cultures; open circles: ethionine-treated cultures; arrow: time of ethionine addition.

If at zero time (defined previously as 72-hr growth) no ethionine is added, the total acceptor activity (defined as the sum of the 20 individually determined specific acceptor activities of a sample) decreases slightly after 20 hr of additional shaking (Figure 4). The loss occurs mainly by reduction of the specific acceptor activity for asparagine, glutamine, and cysteine. If, however, ethionine is added at zero time, then after 8 hr, 30.3% of the total acceptor activity is lost. The reduced level remains unchanged for at least an additional 10 hr (Figure 4). By extrapolation, this total activity value after 10 hr in ethionine would be reached by the untreated sample in 2 days (44–48 hr).

For four of the amino acids (valine, tryptophan, aspartic acid, and phenylalanine), no pattern of significant change in specific acceptor activity occurs whether or not ethionine is added. Fifteen or twenty hours after ethionine treatment the molar per cent of these four accordingly increases. An additional three, previously mentioned (cysteine, asparagine, and glutamine), lose acceptor activity whether or not ethionine is added. Ethionine does specifically stimulate a loss of specific acceptor activity for the remaining 13 amino acids. The time course for one representative of each of these three classes is shown in Figure 5.

Discussion

The degeneracy of the genetic code, the associated tRNA degeneracy, and the critical role of tRNA in protein synthesis suggest, on theoretical grounds, several ways in which tRNA could play a role in the regulation of protein synthesis. The "adaptor-modification" hypothesis has been developed by Sueoka and Kano-Sueoka (1964). A related model might involve the appearance of a new tRNA species preceding differentiation, allowing the translation of a previously untranslatable codeword and thus the synthesis of a new class of proteins. Any such model predicts qualitative and/or quantitative differences in the tRNA taken from an organism

during different physiological or developmental states, although, of course, such differences alone do not prove any role in regulation.

The nature of the changes reported here differs from other reported cases of tRNA alterations accompanying differentiation. Most of these cases involve either the appearance of new tRNA components or shifts in the mobility of preexisting components, *i.e.*, qualitative alterations. Differential transcription of tRNA cistrons (Doi *et al.*, 1966) and enzymatic modification of tRNA (Kano-Sueoka and Sueoka, 1966), respectively, have been hypothesized to account for such observations. The remaining cases of reported tRNA alterations (Kaneko and Doi, 1966; Mushinski and Potter, 1968; Vold and Sypherd, 1968) involve shifts in the relative proportions of multiple tRNA components, similar to the Glu-tRNA shift reported here. However, those cases do not appear to involve any net change in acceptor activity. We have looked only for those tRNA alterations which would be independent of the source of the synthetases. It is possible that aminoacyl-tRNA synthetase alterations occur as well as tRNA alterations and that by using enzyme preparations from exponential cultures, altered tRNA species may have been overlooked.

The following scheme accounts, we believe, for the observations herein reported. Ethionine, added to near-starving, stationary mycelium, stimulates, as part of a complex response to the poor environment, the synthesis of ribonuclease activity and inhibits directly or indirectly RNA synthesis. As a consequence the total RNA content of such mycelium declines.

The sRNA extracted from mycelium at any subsequent time is, presumably, a mixture of intact tRNA molecules, "nicked" molecules, and fragments (which result from denaturation of nicked molecules). Nishimura and Novelli (1963–1965) have directly demonstrated that some tRNA molecules can retain their amino acid acceptor activity after RNase treatment, however, only if the molecules are not allowed to denature and dissociate into fragments.

Up to 8 hr after the addition of ethionine and the resulting acceleration of net tRNA breakdown no change in specific acceptor activity is evident when assayed by the conversion of radioactive amino acids into a trichloroacetic acid insoluble form. The chromatographic evidence, however, shows that changes in specific acceptor activity occur by 5 hr. The contradiction arises, we think, because while RNase-nicked molecules may retain their acceptor activity they do not retain their characteristic chromatographic mobility on MAK. This may be explained by the fact that no Mg^{2+} is present either in the applied sample or during chromatography and under such conditions the molecules may lose their secondary structure (Adams *et al.*, 1967). The nicked molecules dissociate and the resultant fragments do not stick to the column, presumably.

The chromatographic evidence indicates that some tRNA molecules are relatively more resistant to degradation *in vivo* than others, and that the degree of sensitivity, with the exception of glutamine tRNA, is similar for synonymous tRNA acceptors; for example, both valine acceptors have the same sensitivity. This

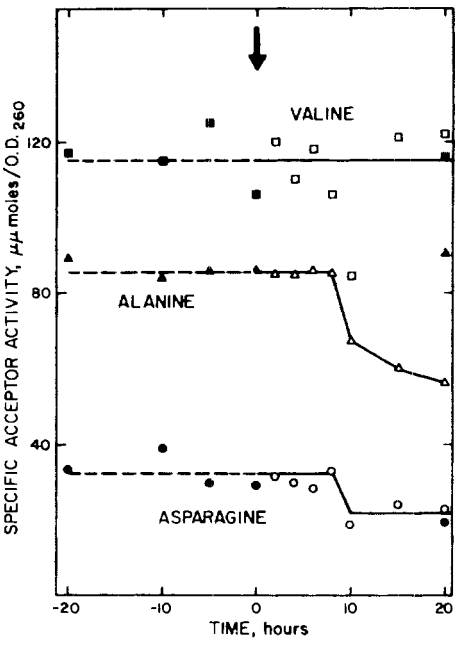

FIGURE 5: Changes in specific acceptor activity for alanine, asparagine, and valine as a function of length of treatment. Alanine (triangles) represents 13 amino acids for which specific acceptor activity declines in response to ethionine. Valine (squares) represents four amino acids, for which the specific acceptor activity remains unchanged. The specific acceptor activity for asparagine (circles) and two other amino acids declines whether or not ethionine is added. Solid symbols: untreated controls; open symbols: ethionine-treated. Arrow: time of ethionine addition.

may reflect a similarity in nucleotide sequence of synonymous acceptors. Such a similarity of sequence is expected since a single enzyme seems to recognize multiple components (Yamane and Sueoka, 1964).

On this hypothesis the changes in specific acceptor activity, assayed by the filter paper disk method, of tRNA taken from cultures treated for 10 hr or longer do not reflect precisely the same parameter that the chromatographic changes reveal. Consequently the aminoacyl-tRNAs which indicate alterations by the two techniques are not necessarily the same nor are their magnitudes. Chromatography reveals relative ribonuclease sensitivity, whereas the direct assay reflects how many nicks each tRNA component can sustain before its acceptor activity is lost. Presumably, some tRNA molecules are sensitive to digestion in that they sustain "hits" but require many of them before losing acceptor activity. This may be simply a question of the proximity of the nuclease-sensitive sequences of the molecule to the region important in determining amino acid acceptance.

For a variety of reasons, it does not seem probable that ethylation of tRNA plays any role in the observed changes of specific acceptor activity. Ethylation of tRNA does occur in rat liver but not in other rat tissues (Farber, 1963) and not in *E. coli* (Smith and Salmon, 1965). The insignificance of tRNA ethylation in *Neurospora* is suggested by direct incorporation experiments and shown indirectly by the fact that even a large concentration of ethionine in the medium reduces tRNA syn-

thesis much more than it affects tRNA methylation. The lack of altered chromatographic mobility for any of the 40 aminoacyl-tRNA components of *Neurospora* also suggests that ethylation does not occur.

For at least three reasons we do not believe that the quantitative changes of *Neurospora* tRNA acceptor activity reported here play a role in regulating the system in which they occur. (1) The relative changes in specific acceptor activity observed are small compared with the large net loss (in milligrams of tRNA per gram of mycelium) of total tRNA. (2) The alterations observed do not generally affect the relative proportion of synonymous tRNAs. (3) Any theory in which tRNA plays a regulatory role must postulate that the availability of tRNA is limiting the rate of protein synthesis. This requirement is apparently not met in this system. After ethionine treatment of mycelium for 5 hr, a time when 40% of the general tRNA content of the mycelium has been lost, there is only a 13% decline in the rate of protein synthesis, as measured by the incorporation of radioactive amino acid, corrected for pool dilution, into a hot trichloroacetic acid insoluble form.

The functional significance of the net breakdown of RNA and soluble protein in this system is unclear. Perhaps the catabolic products are used in the synthesis of other kinds of macromolecules, for example, insoluble proteins, polysaccharides, and/or lipids.

References

Adams, A., Lindahl, T., and Fresco, J. R. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1684.

Baliga, B., Srinivasan, P. R., and Borek, E. (1968), *Federation Proc. 27*, 794.

Chambers, R. W. (1966), sRNA–Amino Acid Acceptance, Technical Brochure 66TR1, Orangeburg, N. Y., Schwarz BioResearch, Inc., p 8.

Doi, R. H., Kaneko, I., and Goehler, B. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1548.

Farber, E. (1963), *Advan. Cancer Res. 7*, 383.

Fling, M., Horowitz, N. H., and Heinemann, S. F. (1963), *J. Biol. Chem. 238*, 2045.

Gartland, W. J., and Sueoka, N. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 948.

Gornall, A. G., Bardawill, C. J., and David, M. W. (1949), *J. Biol. Chem. 177*, 751.

Hirsch, H. M. (1954), *Physiol. Plantarum 7*, 72.

Holland, J. J., Taylor, M. W., and Buck, C. A. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 2437.

Horowitz, N. H. (1965), *Biochem. Biophys. Res. Commun. 18*, 686.

Horowitz, N. H., Fling, M., Macleod, H., and Sueoka, N. (1960), *J. Mol. Biol. 2*, 96.

Horowitz, N. H., Fling, M., Macleod, H., and Watanabe, Y. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 233.

Kaneko, I., and Doi, R. H. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 564.

Kano-Sueoka, T., and Sueoka, N. (1966), *J. Mol. Biol. 20*, 183.

Kappy, M. S., and Metzenberg, R. L. (1965), *Biochim. Biophys. Acta 107*, 425.

Kwan, C. N., Apirion, D., and Schlessinger, D. (1968), *Biochemistry 7*, 427.

Lazzarini, R. A. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 185.

Lee, J. C., and Ingram, V. M. (1967), *Science 158*, 1330.

Mandel, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.

Mushinski, J. F., and Potter, M. (1968), *Federation Proc. 27*, 802.

Nishimura, S., and Novelli, G. D. (1963), *Biochem. Biophys. Res. Commun. 11*, 161.

Nishimura, S., and Novelli, G. D. (1964), *Biochim. Biophys. Acta 80*, 574.

Nishimura, S., and Novelli, G. D. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 178.

Pall, M. L. (1967), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Patterson, M. S., and Greene, R. C. (1965), *Anal. Chem. 37*, 854.

Shearn, A., and Horowitz, N. H. (1969), *Biochemistry 8*, 295 (this issue; preceding paper).

Smith, R. C., and Salmon, W. D. (1965), *J. Bacteriol. 89*, 687.

Subak-Sharpe, H., and Hay, J. (1965), *J. Mol. Biol. 12*, 924.

Sueoka, N., and Kano-Sueoka, T. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 1535.

Sueoka, N., and Yamane, T. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1454.

Taylor, M. W., Granger, G. A., Buck, C. A., and Holland, J. J. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1712.

Urey, J. (1966), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Vogel, H. J. (1956), *Microbiol. Gen. Bull. 13*, 42.

Vold, B. S., and Sypherd, P. S. (1968), *Proc. Natl. Acad. Sci. U. S. 59*, 453.

Waters, L. C., and Novelli, G. D. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 979.

Wevers, W. F., Baguley, B. C., and Ralph, R. K. (1966), *Biochim. Biophys. Acta 123*, 503.

Yamane, T., and Sueoka, N. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1178.

Yang, S. S., and Comb, D. G. (1968), *J. Mol. Biol. 31*, 139.