CHANGES IN NON-PROTEIN NITROGEN METABOLISM DURING TOBACCO MOSAIC VIRUS BIOSYNTHESIS*

By BARRY COMMONER and PHYLLIS M. DIETZ

(From The Henry Shaw School of Botany, Washington University, St. Louis)

(Received for publication, January 7, 1952)

INTRODUCTION

Inoculation of a tobacco leaf with tobacco mosaic virus (TMV) is followed by the synthesis of new protein, part of which is identical with the inoculum itself. It is of considerable interest to the problem of virus biosynthesis to determine what changes in host nitrogen metabolism are associated with this process and to attempt to derive from this information conclusions concerning the metabolic paths involved in virus formation. Such data may also shed some light on the manner in which virus infection leads to development of characteristic symptoms in the host. This paper compares the metabolism of ammonia, free amino acid, and amide nitrogen in normal and TMV-infected tissue from the same leaf.

Material and Methods

Plants used in these studies were grown in large flats under optimal nitrogen nutrition. They attained the size of field-grown plants; areas of mature leaves were of the order of 1 square foot. Nearly full grown leaves (about 20 inches long) were removed from the plant, washed, and split along the midrib. One half of each leaf was inoculated by rubbing with a solution containing 200 μg. of purified TMV (Johnson strain) per ml. of 0.05 M phosphate buffer, pH 7.0. The other half of each leaf was rubbed with pure buffer, and served as the control. The leaf halves were placed in glass-covered moist boxes with the petioles in water and kept at 23°C. and 100 foot-candles of constant illumination from daylight fluorescent lamps. After 24 hours, the leaves were removed, and 12 mm. discs were punched from the intervein areas under sterile conditions. These were washed in several changes of sterile distilled water and placed in sterile Petri dishes containing 40 ml. of ½ strength Vickery's solution (1) or distilled water. Each dish contained a sample of punches with an original wet weight of about 200 mg. During the culture period, the dishes were kept at 23°C. and 100 foot-candles of constant illumination.

Periodically, samples were removed from both infected and control series. The punches were washed in distilled water, stacked, and a sector of the stack (about

* This work was supported by a grant from the American Cancer Society, recommended by the Committee on Growth.

40 mg.) cut out, weighed, and set aside for virus analysis. The remainder of the discs were weighed and used for nitrogen analyses.

Virus Analysis.—The TMV present in the tissue was isolated and determined according to the method previously described (2).

Ammonia Nitrogen.—The remainder of each sample was homogenized at 5–10°C. in 5 ml. of 70 per cent ethanol. The supernatant was removed by centrifugation and dried at 60°C. The dry residue was taken up in 10 ml. of double distilled water, an aliquot distilled with NaOH in a micro Kjeldahl still into saturated boric acid containing methyl red–methylene blue indicator, and titrated with 0.005 N H_2SO_4.

TABLE I

Accuracy of Analyses

Leaf homogenate present	Added ammonium sulfate N	Added amide N	Hydrolysis	N expected	N found	Recovery
	μg.	*μg.*		*μg.*	*μg.*	*per cent*
0	0	240*	No	0	0	—
0	50	240*	No	50	49.0	98
0	0	41‡	No	0	0	—
0	50	41‡	No	50	49.0	98
100 mg.§	0	0	No	—	3.9	—
100 mg.§	0	0	Yes	—	4.5	—
100 mg.§	0	20*	Yes	24.5	23.8	97
100 mg.§	0	60*	Yes	64.5	70.0	109
100 mg.§	0	100*	Yes	104.5	102.2	98
100 mg.§	25	50*	Yes	79.5	78.4	99
100 mg.§	50	25*	Yes	79.5	78.4	99
100 mg.§	50	50*	Yes	104.5	100.0	96

* Asparagine.

‡ Glutamine.

§ All samples containing homogenate were made up with an aliquot of a homogenate from a single virus-infected tobacco leaf prepared in 0.05 M phosphate buffer (pH 7.0). Each aliquot represented 100 mg. wet weight of leaf tissue.

Amide Nitrogen.—An aliquot of the solution used for ammonia determination was made up to 2 N HCl and hydrolyzed at 100°C. for 2 hours. This procedure gives a quantitative yield of ammonia from the amide groups of asparagine and glutamine (3). The amide value was obtained by subtracting the ammonia value previously determined from the total ammonia in the hydrolysate.

Amino Nitrogen.—This was determined by the ninhydrin method of Moore and Stein (4) using a 1 ml. aliquot of the above solution. The solution was freed of ammonia by adding 1 drop of 0.5 N NaOH and bubbling air through it rapidly for 30 minutes. The optical density of the ninhydrin color at 570 mμ was determined in a Coleman Junior spectrophotometer, and converted to average NH_2 values by comparison with a calibration curve based on various concentrations of valine. Amino acid N was calculated by subtracting the amide N value from the NH_2 N value.

Accuracy of the Methods.—Recovery tests (Table I) showed that the nitrogen found by the ammonia and amide methods was within an average of 3 per cent of the amount expected. The ninhydrin method gave a linear relationship between valine concentration and optical density at 570 mμ. Previous investigations had shown that 90 per cent of the ninhydrin-positive compounds in tobacco leaf extracts give optical densities for the ninhydrin color within 12 per cent of that given by valine (5).

EXPERIMENTS AND RESULTS

1. Leaf Discs Cultured in Distilled Water.—A leaf was removed from the plant, split into halves, inoculated, and treated as described above. The results obtained from periodic analyses of leaf discs cultured in distilled water are shown in Fig. 1. Beginning at about 100 hours after inoculation, virus appeared in the infected samples, finally reaching a concentration of about 30 μg. of TMV N per 100 mg. of leaf tissue. No virus was formed in the control samples.

At the start of the experiment, the control series contained relatively little soluble nitrogen. Ammonia and amino acid nitrogen were undetectable, and about 4.5 μg. per 100 mg. of amide N was found. In the next 24 hours, the amide N was replaced by an almost equal amount of amino acid N; this process was reversed between 24 and 110 hours. After this time, amide N went through a second period of decline, reaching zero at 170 hours. It then increased to a maximum at 210 hours, and finally declined for a third time to zero at 235 hours. No amide was present thereafter. As shown in Fig. 1, these fluctuations were matched by approximately reciprocal changes in the amino acid values.

With certain exceptions, the changes in the infected series resembled those of the control. Amino acid changes were almost identical, but significant differences in amide and ammonia N occurred. Although the rapid fluctuations of amide N in the control were generally matched by those of the infected samples, the amide N of the latter tended to remain slightly below that of the control. At the end of the run, both series agreed in containing no detectable amide. The absence of ammonia N in the initial samples persisted until 160 hours after inoculation. A sudden rise in this value then occurred in both infected and control tissue. In the controls, ammonia reached a maximum at 210 hours, declining thereafter until zero was reached at 285 hours. With the exception of one point, this course was generally paralled by the infected tissue, but the ammonia content was significantly below that of the control. Both series agreed in containing no ammonia before 160 hours and after 285 hours.

Fig. 2 shows that the total soluble non-protein nitrogen (NH_3 N + amino acid NH_2 N + 2× amide N) present in control tissue rose to a maximum and finally fell to a level slightly higher than that of the initial sample. Parallel

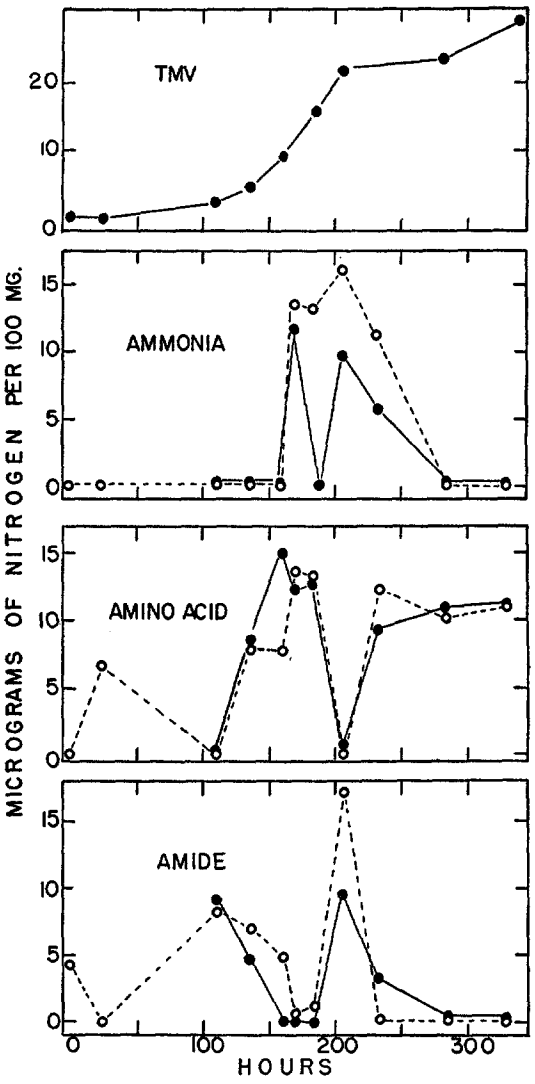

FIG. 1. Changes in TMV, ammonia, amino acid nitrogen, and amide nitrogen of uninfected tobacco leaf discs (broken lines, open circles) and TMV-infected discs (solid lines, closed circles) cultured in distilled water. Infected discs were punched from a half-leaf which had been inoculated with virus at zero hours; uninfected discs were punched from the opposite half of the same leaf which had been rubbed with phosphate buffer at zero time. All analyses were made on aliquots of the same tissue sample.

changes occurred in the infected tissue, but the total soluble N was less than that of the control until 285 hours after inoculation. After this time, the two values agree remarkably. This effect bears a significant relationship to the time-course of TMV formation. Fig. 2 shows that the deficiency in soluble N in the infected tissue as compared with the control rose to a maximum at the time during which TMV was being synthesized. After 210 hours, when TMV

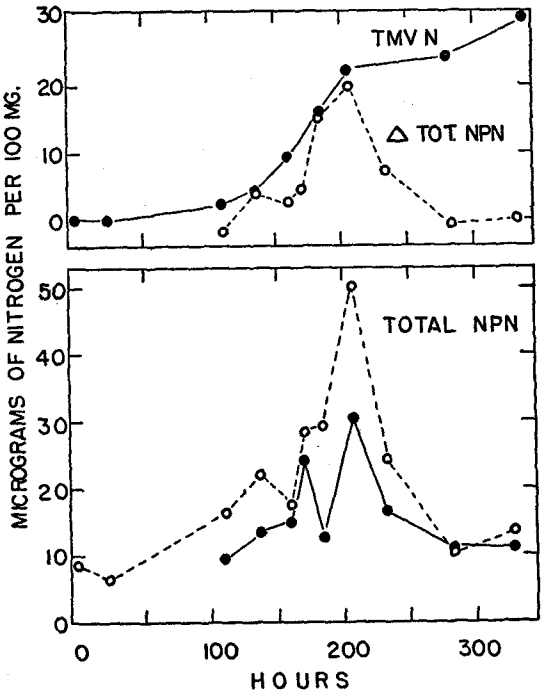

FIG. 2. Data derived from Fig. 1. Total NPN = ammonia N + amino acid N + 2× amide N. △, total NPN = total NPN of uninfected tissue (broken line, lower figure) − total NPN of infected tissue (solid line, lower figure). Abscissa represents hours elapsed since time of inoculation.

formation began to level off, the deficiency declined and finally reached zero. Furthermore, the magnitude of the deficiency, 20 μg. of N per 100 mg. of tissue, is of the order of the maximum amount of TMV N formed (30 μg. per 100 mg.). As will be shown below, these data provide evidence concerning the source of TMV nitrogen.

2. Leaf Discs Cultured in Nutrient Solution.—An experiment identical with the above was carried out with leaf discs cultured in sterile one-half strength Vickery's solution. Since this medium contains ammonia as ammonium sulfate, the tissue was supplied with an abundant outside source of nitrogen.

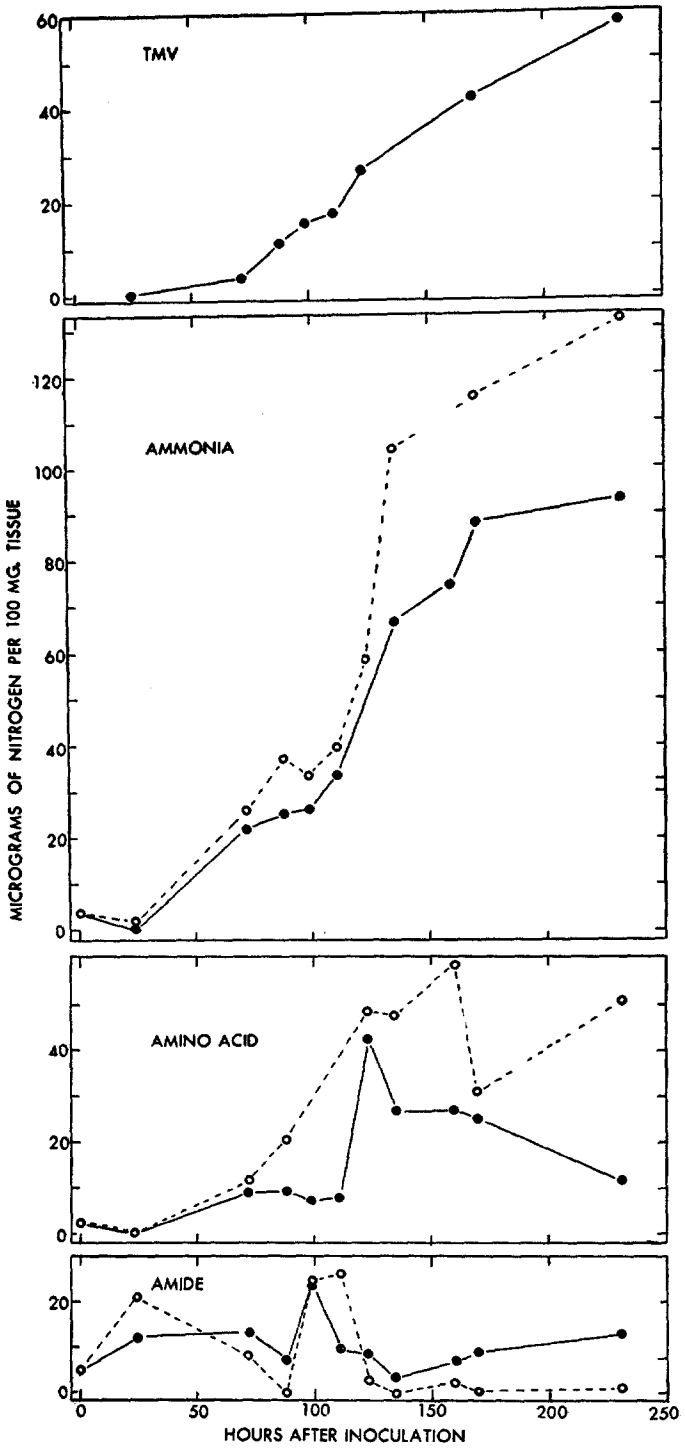

FIG. 3. Changes in TMV, ammonia, amino acid nitrogen, and amide nitrogen of uninfected tobacco leaf discs (broken lines, open circles) and TMV-infected discs (solid lines, closed circles) cultured in a medium containing ammonium sulfate. Other conditions identical with those of the experiment shown in Figs. 1 and 2.

Under these circumstances, considerably more TMV is formed than in distilled water.

The results are shown in Fig. 3. By 210 hours after inoculation, the end of the experimental period, the amount of TMV nitrogen was 55 μg. per 100 mg. This is about twice the amount of virus formed in water culture. In the control series, the amide N rose in the first 24 hours after inoculation, then fell slowly to zero at 90 hours. This was followed by a sharp rise to a maximum of 25 μg. per 100 mg., and a second decline to zero at 124 hours. Amide was

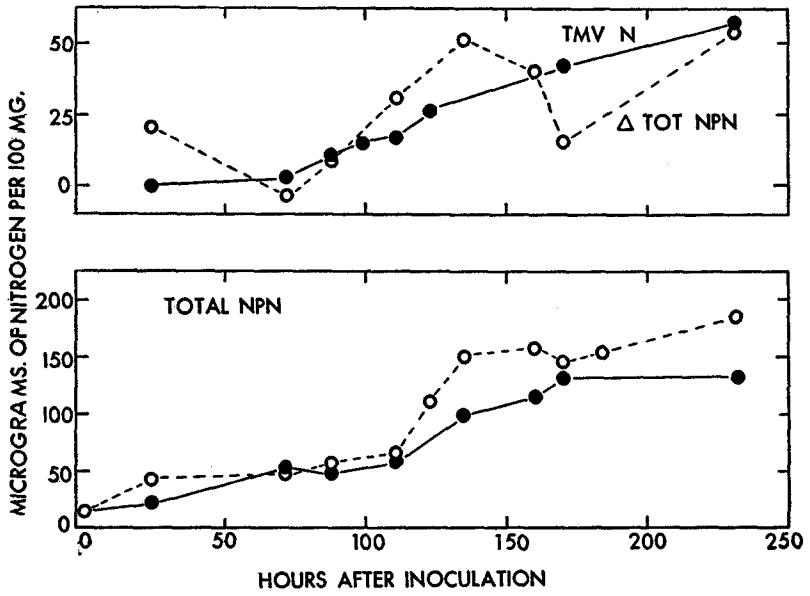

FIG. 4. Data derived from Fig. 3. Total NPN = ammonia N + amino acid N + 2× amide N. △, total NPN = total NPN of uninfected tissue (broken line, lower figure) − total NPN of infected tissue (solid line, lower figure).

absent thereafter. Up to 110 hours, changes in amino acid N tended to reciprocate the amide changes, but this effect is less regular than in the previous experiment. After 110 hours a large amount of amino acid N appeared without a concomitant reduction in amide N. The curves for the infected series are generally similar to those of the control. However, even in the early stages of the infected series, there was practically no reciprocity between amide and amino acid N. At later stages, the infected series had somewhat more amide and less amino acid N than the control.

The curves for ammonia N reflect the availability of external ammonia. Starting at 24 hours after inoculation, the ammonia content rose, levelling off for a short time at 100 hours, and then rising very rapidly to a maximum

of 135 μg. N per 100 mg. at the end of the run. With the exception of a single point, the curve for the infected series showed less ammonia than the controls. This effect was marked after about 120 hours.

Since ammonia represents the major part of the soluble nitrogen in these samples, the total soluble non-protein nitrogen curve is similar to the ammonia curve. As can be seen from Fig. 4, the infected tissue followed the control except that less non-protein nitrogen was present toward the end of the run. This figure also compares this nitrogen deficiency with the amount of nitrogen present as TMV. Although the points are scattered, it is apparent that the deficiency increased as TMV nitrogen accumulated. As in the previous experiment, the deficiency of total amino acid and amide residues in the infected series was considerably less than the concomitant deficiency in total soluble nitrogen.

Thus, comparable results were obtained in both the presence and absence of external nitrogen. Despite the difference in nitrogen level between the two experiments, both showed that TMV formation was accompanied by a deficiency in the pool of soluble nitrogen. In water-culture, this effect halted with the cessation of TMV formation. In the nutrient-cultured tissue, the deficiency persisted to the end of the experiment, apparently because virus formation had not yet stopped at that time.

DISCUSSION

Ammonia, amino acids, and amides account for nearly all of the actively metabolized soluble non-protein nitrogen of the tobacco leaf. This metabolic pool is fed by external nitrogen (ammonia, in the case of our nutrient-cultured material) and by the products of proteolysis. The pool is drained by the incorporation of nitrogen into newly synthesized proteins, including TMV (6).

From a number of investigations (see Street (7)) it is known that regardless of source, the components of the nitrogen pool are subject to a complex series of nitrogen transfers including: reversible deamination of amino acids, reversible deamidization of amides, and transamination between amino and keto acids. Thus the concentration of ammonia, amino acid, and amide N at any given moment represents a steady state resulting from various interacting processes. Changes in the concentrations of these constituents cannot be interpreted in terms of the stoichiometry of any single reaction, for such a relationship will be evident only where the rate of the one process is so much larger than all others, that its effects are not obscured.

The reciprocity between amide and amino acid N discernible in the data needs to be interpreted in this light. In the absence of other processes, the disappearance of amide N and the reciprocal appearance of amino acid N should be equal and accompanied by a corresponding increase in free ammonia. That the reciprocity between amide and amino acid N is neither

quantitative nor associated with ammonia accumulation is evidence that concomitant reactions occur at significant rates.

The results obtained with discs cultured in nutrient containing ammonia show that the external nitrogen source raises the steady state concentrations of all constituents, particularly that of ammonia itself. The rapid entry of external ammonia into the nitrogen pool tends to obscure the reciprocal relationships found in the water-cultured series. In the early phases of the run, when ammonia absorption is relatively slow, the reciprocity between amino acid and amide N is evident, particularly in the uninfected series. Later, the rapid influx of ammonia obliterates this relationship and amino acid N accumulates without reduction in amide N.

Both experiments show characteristic fluctuations in the composition of the nitrogen pool. That these are real is indicated by the following: the fluctuations are considerably larger than the analytical errors; they are remarkably similar in opposite leaf-halves; independent analyses of protein metabolism in cultured leaf discs by electrophoresis (8) and by Kjeldahl N determinations (6) show corresponding fluctuations in protein composition. Apparently the multiplicity of interactions between leaf nitrogen components leads to overshoot phenomena. Certain fluctuations, especially those of ammonia and amide, appear to involve periods of about 100 hours. Changes in protein and water content, which also follow this periodicity, have been observed (6).

In both series, infection with TMV results in no significant differences in the *general* pattern of changes in concentration of the soluble nitrogenous constituents. However, regardless of the level of nitrogen metabolism, TMV biosynthesis is associated with a reduction in the size of the pool. Since this deficiency coincides both in time and in order of magnitude with the formation of new virus protein, it is reasonable to conclude that it results from a transfer of soluble N from the metabolic pool to the TMV protein. Since a large part of this deficiency is due to ammonia, synthesis of the bulk of the TMV protein must occur *de novo* from ammonia and nitrogen-free carbon residues rather than by condensation of intact amino acid and amide residues. The conclusion that the major part of TMV nitrogen is derived from the soluble components of the metabolic pool is consistent with the results of N^{15} tracer studies by Meneghini and Delwiche (9) and by Commoner and Schieber (10).

Our data also show that the effects of virus infection on the metabolism of the host are due to the synthesis of TMV, rather than to its presence. This is apparent from (*a*) the observation that the general pattern of changes in composition of the nitrogen pool during the culture period is quite similar in both uninfected and infected leaf-halves; and (*b*) the facts that the reduction in the level of the pool ends with the cessation of virus synthesis, and that the metabolic equilibria occurring in the control tissue are then quickly reestablished in the infected material. Thus, the observed effects of TMV

on the host appear to be a consequence of the virus' sole biological property: reduplication.

These results suggest that the major qualitative effects on host metabolism caused by entry of the virus occur in the mechanisms of protein synthesis. Reduplication of TMV appears to be a result of the virus' unique ability to induce the host's protein-synthesizing machinery to yield a product identical with the inoculum.

SUMMARY

1. Discs cut from tobacco leaf tissue infected with tobacco mosaic virus and cultured in water contain less non-protein nitrogen than comparable uninfected discs during the time at which TMV is formed. This deficiency disappears when virus formation ceases. Discs cultured in nutrient solution form about twice as much TMV as discs cultured in water. The maximum non-protein nitrogen deficiency is comparable in magnitude to the amount of virus synthesized.

2. The largest difference between infected and uninfected tissue occurs in the ammonia content. Smaller, but significant differences in amide content are found. Infected discs cultured in water show no significant differences from control discs in free amino acid content; infected discs cultured in nutrient solution develop a small deficiency in amino acid nitrogen.

3. The general patterns of change in composition of the pool of soluble nitrogen are similar in both infected and uninfected discs.

4. The data indicate that the bulk of the nitrogen incorporated into virus protein is withdrawn from the leaf's pool of soluble nitrogen; virus is formed *de novo* from ammonia nitrogen and non-nitrogenous carbon sources. The effect of virus infection on host nitrogen metabolism appears to be due to the formation of virus rather than to its presence.

BIBLIOGRAPHY

1. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Bull. Conn. Agric. Exp. Sta., No. 399*, 1937.
2. Commoner, B., Mercer, F. L., Merrill, P., and Zimmer, A. J., *Arch. Biochem. and Biophysics*, 1950, **27**, 271.
3. Chibnall, A. C., Protein Metabolism in the Plant, New Haven, Yale University Press, 1939.
4. Moore, S., and Stein, W. H., *J. Biol. Chem.*, 1948, **176**, 367.
5. Commoner, B., and Nehari, V., data to be published.
6. Commoner, B., and Dietz, P. M., data to be published.
7. Street, H. E., *Advances Enzymol.*, 1949, **9**, 391.
8. Commoner, B., Newmark, P., and Rodenberg, S. D., *Arch. Biochem. and Biophysics*, 1952, **37**, 15.
9. Meneghini, M., and Delwiche, C. C., *J. Biol. Chem.*, 1951, **189**, 177.
10. Commoner, B., and Schieber, D., data to be published.