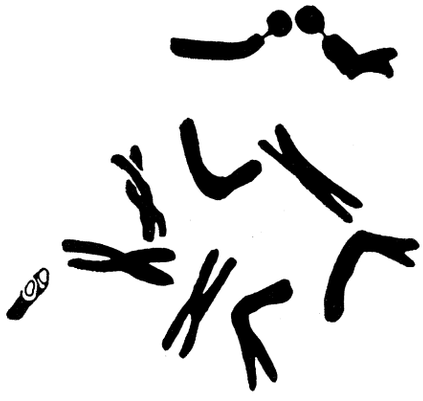

Fig. 1. Chromosomes in the root tip of *T. hirtum*. One pair of chromosomes has satellites. Drawings made with aid of camera lucida. (About × 2200)

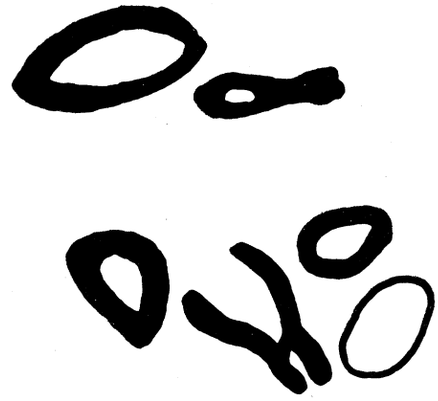

Fig. 2. Chromosomes in meiosis in *Trifolium hirtum*, at the stage of diakinesis. The nucleolus is shown in outline. (About × 1600)

portant clovers described by Hermann (*3*), *T. hirtum* is the only species for which the chromosome number has not been published previously. Cytological studies of *T. hirtum* have now revealed that its somatic chromosome number is 10 (Fig. 1) and that one pair of the chromosomes has satellites (*4*). Preparations of chromosomes were made from root tips which had been treated with 0.002*M* hydroxyquinoline for 2 hours at 4°C prior to fixing. Five different accessions of seed were examined.

A chromosome number of 10 is unusual for the genus: out of approximately 70 species of *Trifolium* for which the chromosome numbers are known, only one other, *T. scabrum* L., has this number. All *Trifolium* species for which the chromosome numbers were reliably counted prior to 1952 were reported to have basic numbers of 7 or 8. In that year Yates and Brittan (*5*) found a chromosome number of $2n = 12$ for some races of *T. subterraneum* L. The 12-chromosome clovers were subsequently separated from the 16-chromosome *T. subterraneum* on the basis of morphological differences, and were designated *T. israeliticum* D. Zoh. and K. (*6*). No other 12-chromosome clover has yet been reported.

Larsen (*7*) reported another new chromosome number in 1960 when he found *T. scabrum* L. to have $2n = 10$ chromosomes. This species was previously reported to have 16 chromosomes (*8*), but the 10-chromosome number has been confirmed independently (*9*). The only other clover found with a possible basic number of 5 is a form of *T. lupinaster* L. with $2n = 40$ chromosomes (*10*). Other forms of *T. lupinaster* were reported by Karpechenko (*8*) and Il'in and Trukhaleva (*10*) to have 48 and 32 chromosomes. Considering these facts, as well as taxonomic position, a form of *T. lupinaster* with a base number of 5 chromosomes does not appear to be probable. Taxonomically, both *T. hirtum* and *T. scabrum* are in the subgenus Trifolium of Hossain (*11*). *Trifolium lupinaster* is in the subgenus Amoria.

Trifolium hirtum is, then, the second species of *Trifolium* found to have 10 chromosomes (*12*).

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References and Notes

1. R. M. Love and D. C. Sumner, *Calif. Agr. Exptl. Sta. Circ. No. 407* (1955).
2. W. A. Williams, R. M. Love, L. J. Berry, *ibid.* 458 (1957).
3. F. J. Hermann, *U.S. Dept. Agr., Agr. Monograph No. 22*, (1953), p. 1.
4. Seed kindly supplied by Dr. W. A. Williams, University of California, Davis.
5. J. J. Yates, N. H. Brittan, *Australian J. Agr. Res.* **3**, 300 (1952).
6. D. Zohary, J. Katznelson, *Australian J. Botany* **6**, 177 (1958).
7. K. Larsen, *Danske videnskabernes selskab Biologiske skrifter No. 11* (1960), p. 1.
8. G. D. Karpechenko, *Bull. Appl. Botan. Plant Breeding (Leningrad)* **14**, 271 (1925); J. E. de Mesquita Rodrigues, thesis, Univ. of Coimbra (1953), pp. 87–88.
9. E. Kliphuis. *Acta Botan. Neerl.* **11**, 90 (1962).
10. M. M. Il'in and N. A. Trukhaleva, *Dokl. Botan. Sci. Sect. (English Transl.)* **132**, 107 (1960).
11. M. Hossain, *Notes Roy. Botan. Garden Edinburgh, No. 23* (1960), p. 387.
12. Published with the approval of the Director of the Hawaii Agricultural Experiment Station as Technical Note No. 1.

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Protein Biosynthesis: Some Alternative Considerations on the Current Hypothesis

Several recent articles give the impression that the problem of protein biosynthesis is largely solved and that the remaining unsettled questions concern specific details (*1*, *2*). The purpose of this discussion is to emphasize that the current formulation still represents a working hypothesis. New findings which do not readily fit in the current visualization should be used to help modify the hypothesis. It is very important at this stage of our understanding to be careful not to select only those findings and interpretations which are consistent.

The essential features of the most generally accepted mode of protein synthesis are as follows.

1) All of the information for making specific proteins is contained in a linear sequence of nucleotides.

2) The information is written in the form of a code which uses 3 (or a multiple of 3) nucleotides to specify a single amino acid. The information may be carried predominantly in two of three nucleotides (*2*, *3*).

3) An exact replica of the DNA sequence is made in the form of RNA with uracil substituting for thymine.

4) This replica called "messenger RNA" attaches itself to ribosomes already existing in the cytoplasm and from this location directs the formation of protein.

5) Amino acid is activated for protein synthesis, and in chemical association with a specific molecule of soluble ribonucleic acid it is brought to a particular location on the messenger. This location recognizes the soluble ribonucleic acid and not the amino acid.

6) The messenger has a short life (2 to 3 minutes) and, after making several proteins, it is degraded. More recently, in order to retain the concept of messenger in those cases where evidence for a rapidly renewed message was not obtained, the possibility of a more stable message is considered.

In regard to point 1, the evidence which demonstrates that nucleic acid is the carrier of genetic information is firmly supported by experiments on bacterial transformation, infection of hosts by virus nucleic acid, and induced and natural mutations which affect the nucleic acids and result in specific substitutions of amino acids. That nucleic

acid is the only participant in the storage and transfer of information is a satisfying but not unique interpretation of these experiments. Another tenable explanation could be that genetic information is written in the form of a polynucleotide in juxtaposition to a nonnucleotide polymer existing in the host cell. Reservations of this kind have recently been stated (*4*).

In regard to point 2, it is worth remembering that the origin of the triplet concept was the result of a logical process and was not based on the interpretation of experimental data. If all of the information is carried in the linear sequence, then it is obvious that if only one nucleotide acted as a code for one amino acid, the four nucleotides would be insufficient for 20 amino acids. By similar reasoning, since two nucleotides alone would act as a code to $4 \times 4 = 16$ amino acids (an insufficient number), then at least three nucleotides would be needed since $4 \times 4 \times 4 = 64$, (see also *2* and *3*).

In an analysis of acridine-induced mutations in *Escherichia coli* infected with T4 phage, Crick *et al.* (*5*) provided evidence obtained in a study of changes in the B cistron of the R_{II} region of the phage which lend support to the triplet hypothesis.

Point 5 (above) concerns the role of sRNA in protein synthesis. This subject has been treated in some detail (*6*) and will not be considered further. However, it should be noted that according to the scheme as now conceived, the role of sRNA is directly linked to the function of messenger RNA. The fact that some question exists on the universality of the requirement for sRNA in processes of protein synthesis means that the same question would have to apply to the role of messenger in this scheme.

Points 3 to 6 comprise the messenger RNA concept, and the discussion which follows is concerned mainly with this subject. The modern idea of the messenger was emphatically stated with supporting experimental data by Brenner, Jacob, and Meselson (*7*), although the idea was originally discussed by Jacob and Monod (*8*). Since this statement launched the existing messenger concept and is often referred to, it is considered here in some detail. The support for the messenger concept from Brenner *et al.* was of three kinds, namely, completely theoretical, logical, and experimental. The theoretical considerations were discussed in the first paragraph. The points raised are not particularly compelling and alternative explanations could easily be proposed. The rest of the argument suffers from two very serious weaknesses.

First, three models are considered to represent the process of normal protein synthesis and the changes that take place during phage infection. Experimental data were interpreted as being incompatible with two of these. Since, however, these three models in no way exhaust the possibilities for representing the events under study, the fact that data are incompatible with two of the three models does not strengthen the case for the third, even though these data may be compatible with this third model.

Second, an integral feature built into all three models is the assumption that the Volkin and Astrachan type of RNA (*9*) participates in the transfer of information from DNA to the protein-synthesizing system. That this assumption does not uniquely follow from the experimental observations is demonstrated by the fact that a completely different explanation was considered when Astrachan and Volkin discussed their own original findings (*10*). Furthermore, newer observations with uninfected cells would suggest that much of this type of RNA functions as an early precursor stage in the formation of stable ribosome (*11*, *12*).

The problems associated with evaluating the data in terms of only three models can best be demonstrated by interpreting the experimental observation in terms of a fourth model. In this model, the active site of protein synthesis is considered to be not the free ribosome, but a membrane-associated ribosome. This concept actually accounts for many published observations (*6*). More recent experiments suggest that in *E. coli* such a model might apply (*13*). In this model, the Volkin and Astrachan type RNA is considered as an early stage of ribosome formation. The experiments performed by Brenner *et al.* showed that (i) the new C^{14}-uracil-labeled RNA formed after phage infection was associated with the ribosome band assumed to be of the 70*S* class; (ii) this RNA was metabolically turning over since after 16 minutes growth in C^{12}-labeled medium diluted 200 fold, only 25 percent of the radioactivity was still associated with the 70*S* ribosomes; (iii) if cells were grown in N^{15}–C^{13}-labeled medium, infected with T4 bacteriophage, transferred to N^{14}–C^{12}-labeled medium, and fed P^{32}-labeled phosphate from the 2nd to the 7th minute, the P^{32} was associated mainly with the N^{15}–C^{13}-labeled 70*S* ribosomes although there was some associated with the lighter ribosomes. Their interpretation was that the new RNA was associated only with already existing ribosomes and that no wholly new ribosomes were synthesized after phage infection. However, a completely different interpretation could readily be offered on the basis of the fourth model. That is, ribosomes are assembled on the surface of the cell membrane. Therefore, the host cells grown in N^{15}–C^{13}-labeled medium contain a whole chain of N^{15}–C^{13}-labeled ribosome precursors. The new RNA is incorporated into the new ribosomes being synthesized at the cell membrane and released during the process of cell disruption. The N^{14}–C^{12}-labeled components enter the ribosome-synthesizing sequence from the beginning and in the 5 minutes of growth in P^{32}-labeled medium, a small percentage of N^{14}–C^{12}–P^{32}-labeled ribosomes would be the result of a continuous production of new ribosomes containing the new RNA which serves as ribosome precursor. It should be noted that this interpretation is compatible with the recent findings cited above (*11*, *12*).

In an experiment to determine if new ribosomes or those already existing make protein, a similar approach was used by Brenner *et al.* (*7*). Cells were grown in N^{15}-labeled medium, infected with phage, transferred to N^{14}-labeled medium, and fed S^{35}-labeled sulfate for the first 2 minutes of growth. The result was that only N^{15}-labeled ribosomes contained S^{35}. Their interpretation was that protein was synthesized only on already existing ribosomes. According to model four, however, protein was made on the ribosomes newly synthesized from the N^{15}-labeled precursors or already existing on or in the cell membrane.

An important facet in the theoretical development of the messenger concept regarding synthesis of protein in bacteria is the high rate of metabolic turnover of the messenger molecule. This is dictated because of the fact that the kinetics of enzyme induction and repression are quite rapid; therefore the message must be quickly renewed or destroyed. Furthermore, as pointed out

by Gros *et al.* (*14*), the amount of bacterial RNA, which could be messenger, is only a few percent at most. For pulse labeling of RNA to reflect base compositions of DNA, an appreciable percentage of the newly synthesized RNA must be messenger. Since the steady-state amount of messenger RNA would be only a few percent of the total, it follows that the DNA-like RNA must be turning over very much faster than the bulk RNA. Indeed, the experimental results of Brenner *et al.* (*7*) and Gros *et al.* (*14*) seem to confirm this property of rapidly turning over messenger RNA which transiently was associated with fully formed 70*S* or 100*S* ribosomes in *E. coli*.

Support for this interpretation was also drawn from the fact that a similar DNA-like RNA (D-RNA) in yeast appeared to be rapidly turning over. More recent information, however, with both the *E. coli* D-RNA (*11*) and yeast D-RNA (*12*) give a somewhat different picture. Ycas and Vincent, who originally described the D-RNA of yeast, in later experiments with Kitazume (*12*), reported that there was no obvious relationship between the synthesis of D-RNA and that of protein. They further reported that D-RNA was an obligatory precursor to the bulk of cellular RNA and therefore, that it was not being extensively degraded such as is thought to be the case with messenger. These findings confirm those of McCarthy, Britten, and Roberts (*11*) who showed that the RNA fraction in *E. coli* which has the properties of the messenger RNA functions largely as a precursor to the ribosomes and that no evidence was obtained to support the contention of Gros *et al.* and Brenner *et al.* that this fraction of messenger RNA was being rapidly and extensively degraded to smaller stages. Tissieres and Watson (*15*) studied the attachment of "messenger RNA" in vivo to ribosomes and its concomitant breakdown linked to amino acid incorporation. They calculated that about 17 μg of messenger RNA was broken down for each 1 μg of amino acid incorporated (that is, 4 to 5 moles of nucleotides per mole of amino acid). On the other hand, using synthetic messenger RNA, Barondes and Nirenberg (*16*) showed that in vitro about 80 percent of the polynucleotide was rapidly broken down before C^{14}-phenylalanine incorporation reached its maximal rate and that during the phase of active amino acid incorporation, the nucleotide breakdown was at a minimal rate. That is, approximately 2 μmole of amino acid were incorporated for each micromole of nucleotide released. It is important to mention that in both of these studies, the net disappearance of messenger was followed. A concomitant resynthesis, if it occurred, would complicate the interpretation.

Two very interesting papers have recently appeared which are concerned with the same problem of the dependence of protein synthesis on the presence of a metabolically unstable RNA messenger (*17*, *18*). Both of the reports concerned the effects of Actinomycin D with *Bacillus subtilis* in vivo on the inhibition of RNA synthesis and protein synthesis. Hurwitz *et al.* (*17*) found that actinomycin (0.2 μM), inhibited DNA synthesis only 25 percent but inhibited RNA synthesis 90 to 95 percent. The small amount of RNA synthesized (2 to 8 percent), when examined after pulse labeling with P^{32}, did not resemble in base composition either DNA or RNA of *B. subtilis*. Under these conditions, with messenger RNA synthesis completely blocked, protein synthesis continued at 25 to 50 percent of the normal rate. Hurwitz *et al.* concluded that, obviously, protein synthesis independent of unstable messenger RNA must proceed in this organism.

Levinthal *et al.* (*18*), using Actinomycin D at a concentration 40 times greater than that of Hurwitz *et al.*, found that synthesis of protein, as well as of RNA, was completely blocked. Since, however, at the lower concentration a separation of the two inhibitory effects was apparently achieved, the double effect at the higher level cannot be interpreted in terms of a link between the two processes. The foregoing studies do not fit into a single clear picture of messenger activity. This fact is of further concern since the conflicting findings were made on very similar systems.

An essential type of evidence for the firm establishment of the messenger concept, as we now know it, would be the demonstration that a protein-synthesizing system of one species could be induced to make a specific protein characteristic of a completely different species, by the addition of RNA characteristic of the second species. One attempt in this direction was recently described (*19*). Ribonucleic acid of tobacco mosaic virus was added to the *E. coli* system of Nirenberg, Matthaei, and Jones (*20*), and an appreciable stimulation of amino acid incorporation was noted. An evaluation of the findings in relation to the controls shows that of the induced incorporation of amino acid, 9 percent at most could possibly be tobacco mosaic virus (TMV) protein. This amount of protein would, however, be sufficient to establish the point, if it were demonstrated unequivocally to be TMV protein. Although there were some definite similarities between a part of the induced protein and TMV coat protein, no identity was established. The data were obtained by fractionation on Dowex 1 of peptides obtained from a trypsin digest of the purified induced TMV-like protein. The doubt about this kind of proof is reflected by the fact that after incubation with either C^{14}-labeled phenylalanine or tyrosine, peptide peaks not supposed to contain either of these amino acids contained appreciable radioactivity. It is true, of course, that other TMV-induced proteins could be blurring the picture, but this point must be resolved. A peptide "fingerprint" (chromatographic and electrophoretic analysis) would provide a more powerful demonstration of the formation of the TMV-protein.

The beautiful demonstration by Nathans *et al.* (*21*) of the induced formation of f-2 coat proteins in an *E. coli* system does not serve to resolve this point. This phage is an RNA-phage of *E. coli*. There is a valid question as to how much information for the synthesis of f-2 coat protein could be stored in *E. coli*. Nathans *et al.* described control experiments with RNA from TMV in this system. With the techniques used to study the formation of f-2 coat protein, these authors possess the facilities to provide the much-needed information concerning the possible formation of TMV-coat protein. A more recent attempt to identify a polynucleotide-stimulated formation of protein was reported as negative (*22*).

There are other unsettled questions which are not treated in the present scheme of protein synthesis. What happens in the case of synthesis of protein and specific enzymes in enucleated

cells of algae and amebae (*23*)? In the model system of Nirenberg and his associates, single strandedness appears to be an essential feature for messenger activity; that is, polyU (polyuridylic acid) is active, but when polyA (polyadenylic acid) is added along with it, the polyU is ineffective (*24*). From the fact that the base ratio of messenger RNA is supposed to be similar to that of DNA, it would appear that both complementary strands of the DNA are copied and are present. If so, how then does the accepted scheme solve the problem of removing the unwanted strand?

An alternative possibility would be that each single strand of the DNA had a base pair relation of adenine = thymine and guanine = cytosine. In the absence of any reason for such a postulation (such as base-pairing in the case of double strands) this possibility is not attractive at the present time. It should be noted that the situation does not appear to apply for the DNA which is supposed to serve as a template for ribosomal RNA (*25*). Another discussion of the limitations of the current theory in terms of experimental data derived with HeLa cells has recently appeared (*26*).

In conclusion, certain points should be clearly stated. This paper is not an attempt to disprove the validity of the messenger concept. The messenger concept is a very powerful tool with which to view new data and plan further experimental approaches. The messenger concept is, at the same time, only a working hypothesis and one which no doubt will have to be altered as more information is acquired. A potentially undesirable situation is developing, where the distinction between working hypothesis and fact is becoming diffuse. The process of protein synthesis with respect to its direction, control, utilization of energy, and integration with the rest of the cell's activities is not likely to be completely understood until the various implicated reactions are viewed in their relation to cellular organization. The problem should be approached in many different ways. Simple schemes representing our current thinking should not be treated as established patterns.

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References

1. P. C. Zamecnik, *Biochem. J.* **85**, 257 (1962); M. Calvin, *Am. Inst. Biol. Sci. Bull.* **13**, 29 (1962); J. D. Watson, *Science* **140**, 17 (1963).
2. F. H. C. Crick, in *Progress in Nucleic Acid Research* (Academic Press, New York, 1963).
3. R. B. Roberts, *Proc. Natl. Acad. Sci. U.S.* **48**, 1245 (1962); R. W. Eck, *Science* **140**, 477 (1963).
4. N. W. Pirie, *Nature* **197**, 568 (1963); J. R. S. Fincham, *Ann. Rev. Biochem.* **38**, 343 (1959).
5. F. H. C. Crick, L. Barnett, S. Brenner, R. J. Watts-Tobin, *Nature* **192**, 1227 (1962).
6. R. W. Hendler, *ibid.* **193**, 821 (1962).
7. S. Brenner, F. Jacob, M. Meselson, *ibid.* **190**, 576 (1961).
8. F. Jacob and J. Monod, *J. Mol. Biol.* **3**, 318 (1961).
9. E. Volkin and L. Astrachan, in *The Chemical Basis of Heredity* (Johns Hopkins Press, Baltimore, 1957), p. 686.
10. L. Astrachan and E. Volkin, *Biochim. Biophys. Acta* **29**, 536 (1958).
11. J. McCarthy, R. J. Britten, R. B. Roberts, *Biophys. J.* **2**, 57 (1962).
12. Y. Kitazume, M. Ycas, W. S. Vincent, *Proc. Natl. Acad. Sci. U.S.* **48**, 265 (1962).
13. R. W. Hendler and J. Tani, in preparation.
14. F. Gros, H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, J. D. Watson, *Nature* **190**, 581 (1962).
15. A. Tissieres and J. D. Watson, *Proc. Natl. Acad. Sci. U.S.* **48**, 1061 (1962).
16. S. H. Barondes and M. W. Nirenberg, *Science* **138**, 810 (1962).
17. J. Hurwitz, J. J. Furth, M. Malamy, M. Alexander, *Proc. Natl. Acad. Sci. U.S.* **48**, 1222 (1962).
18. C. Levinthal, A. Keynanaand, A. Higa, *ibid.*, p. 1631.
19. A. Tsugita, H. Fraenkel-Conrat, M. W. Nirenberg, J. H. Matthaei, *ibid.*, p. 816.
20. M. W. Nirenberg, J. H. Matthaei, O. W. Jones, *ibid.*, p. 104.
21. D. Nathans, G. Notani, J. H. Schwartz, N. D. Zinder, *ibid.*, p. 1424.
22. B. R. Chatterjee and R. P. Williams, *Biochem. Biophys. Res. Commun.* **9**, 72 (1962).
23. H. Chantrenne, in *The Biosynthesis of Proteins* (Pergamon, New York, 1962).
24. M. W. Nirenberg and J. H. Matthaei, *Proc. Natl. Acad. Sci. U.S.* **47**, 1588 (1962); M. F. Singer, O. W. Jones, M. W. Nirenberg, *ibid.* **49**, 392 (1963).
25. S. A. Yankofsky and S. Spiegelman, *ibid.* **48**, 1466 (1962).
26. H. Harris, H. W. Fisher, A. Rodgers, T. Spencer, J. W. Watts, *Proc. Roy. Soc. London, Ser. B* **154**, 177 (1963).

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Simazine: Degradation by Soil Microorganisms

Abstract: *A soil fungus,* Aspergillus fumigatus *Fres., is effective in the degradation of the herbicide 2-chloro-4,6-bis(ethylamino)-s-triazine (simazine). The degradation of both ring- and chain-labeled (C^{14}) simazine was observed in an unamended and an amended (sucrose) basal medium. A loss of C^{14} occurred in all culture solutions containing either ring- or chain-labeled simazine, but the decrease in activity observed was greater with chain-labeled than with ring-labeled simazine. Chromatographic evidence indicates that* A. fumigatus *may possess a degradation mechanism unlike that which occurs in corn plants.*

The herbicide 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine) is used for selective weed control in crops. Although several investigators (*1*) have suggested that soil microorganisms are at least partly responsible for the degradation of simazine in soil, it has not been clearly demonstrated that soil microorganisms can utilize simazine as a source of carbon or nitrogen. The nature of the metabolic products from the degradation of simazine by soil microorganisms is unknown, but in some higher plants 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine (hydroxysimazine) has been detected as a major metabolic product (*2*, *3*).

In the present investigation soil microorganisms that could degrade simazine were isolated by enrichment of a soil solution. Organisms utilizing simazine as a sole or supplemental source of carbon were *Aspergillus flavipes* (Bainier and A. Sartory) Thom and Church, *A. fumigatus* Fres., *A. ustus* (Bainier) Thom and Church, *Fusarium moniliforme* Sheldon, *F. oxysporum* Schlect., *Penicillium purpurogenum* Stoll, *P.* sp., *Rhizopus stolonifer* (Ehr. ex Fr.) Vuill, *Stachybotrys* sp., *Trichoderma viride* (Pers. ex Fr.), three species of *Streptomyces,* and four bacterial isolates believed to belong in the genus *Arthrobacter.* In comparative studies *Aspergillus fumigatus,* an organism not previously reported by other workers, was most effective in the degradation of simazine. The objective of this investigation was to observe, in an amended and an unamended basal medium with pure cultures of *A. fumigatus,* the degradation of both ring- and chain-labeled (C^{14}) simazine.

The unamended basal medium contained 0.2 g of K_2HPO_4, 0.3 g of NH_4NO_3, 0.2 g of $CaSO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 1 mg of $FeSO_4 \cdot 7H_2O$ in 1000 ml of distilled H_2O. The amended basal medium contained 0.1 g of sucrose as a supplemental carbon source. Sufficient amounts of simazine (*4*) labeled with C^{14} in the chain or ring (specific activity 4.97 and 5.06 μc/mg, respectively) were added to these media so that the