*ALLELIC STRAINS OF NEUROSPORA LACKING TRYPTOPHAN SYNTHETASE: A PRELIMINARY IMMUNOCHEMICAL CHARACTERIZATION**

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The action of genetic material and its relation to enzyme biosynthesis has only in recent years received attention from an experimental standpoint. A variety of enzyme changes has been found to be associated with genetic alterations, including the formation of altered enzymes, the modifications in various quantitative aspects of enzyme formation, and the complete loss of ability to form specific enzymes (see review[1]). The first two categories have received some attention in previous investigations. The last category, which might be the most informative, has received scant attention in the past, largely because the lack of enzyme activity severely limits a direct experimental approach. However, the absence of detectable enzyme activity need not abolish all hope of examining such a condition. Even if the ability to form a specific enzyme were lost, there might remain the synthesis of proteins antigenically similar to that enzyme, and the presence of these could be investigated by immunochemical methods. This approach has recently been employed in studies of the β-galactosidase system of *Escherichia coli*[2] and of the tyrosinase of *Glomerella*.[3]

The present paper is a preliminary report on the application of immunochemical techniques to problems of gene action and of the control of tryptophan synthetase formation in *Neurospora crassa*. This enzyme was selected for study because a series of well-characterized alleles affecting its formation was available,[4, 5] as well as detailed information about the enzyme itself.[6, 7, 8]

Materials and Methods.—The various tryptophan independent (T^+) and tryptophan requiring (T^-) strains of *N. crassa* used in this work are listed in Table 1.

TABLE 1

STRAINS OF *N. crassa* EMPLOYED IN THE IMMUNOLOGICAL EXPERIMENTS

Strain	Tryptophan Requirement
Wild type (5256 A)	Tryptophan independent (T^+)
td_1, td_2, td_3, td_6, td_7	Tryptophan requiring (T^-)
td_1su_2	Tryptophan requiring (T^-)
td_2su_2, td_3su_3, td_6su_6	Tryptophan independent (T^+)

The tryptophan requiring strains employed, td_1, td_2, td_3, td_6, and td_7,[4, 5] represent independent mutations affecting the ability to convert indole to tryptophan. These mutants specifically lack tryptophan synthetase.[4, 5, 7] Genetic tests have indicated that these strains represent mutations of the same locus or of closely linked interdependent genetic material.[4, 5, 9] The tryptophan independent T^+ strains employed were of two types: parental strains and suppressed mutant strains (td su). As previously reported,[4, 5] suppressor mutations restore to specific td mutants the ability to grow in the absence of tryptophan and to form detectable amounts of tryptophan synthetase.

T^+ cultures were grown with aeration in 20 liters of glucose (1 per cent) minimal medium[10] for 60–70 hours at 30° C.; T^- cultures were grown in the presence of a supplement of 3 gm. of DL-tryptophan. The preparation, purification, and method of assay of extracts have previously been described.[8] The tryptophan synthetase unit (TSU) employed is the amount of enzyme which will catalyze the conversion of 0.1 μ mole of indole to tryptophan in a 60-minute incubation at 37° in the presence of saturating concentrations of indole, serine, and pyridoxal phosphate. Protein was determined by the methods of Lowry[11] and of Robinson *et al.*[12]

For immunization purposes an eightfold purified preparation of tryptophan synthetase was used as antigen. Four rabbits received approximately the same total dose of enzyme, 150 tryptophan synthetase units in about 45 mg. of *N. crassa* protein. All the rabbits were bled before receiving enzyme and again 6 weeks after the first injection. Inactivation of tryptophan synthetase by normal serum does not occur during incubation periods up to 1 hour. Normal serum inactivation of tryptophan synthetase does become a problem, however, if long incubation periods are used. This problem can be overcome by the use of sera which have been heat-treated and fractionated by the ammonium sulfate method of Tiselius.[13] Thus, for incubation periods up to 1 hour, whole untreated sera could be used. For long incubation periods, fractionated, heat-treated (56° C. for 30 minutes) sera were employed.

Precipitin reactions were ordinarily carried out at 5° C. At various times aliquots were removed, centrifuged in the cold, and carefully decanted. Enzyme determinations were routinely made on the supernatant solutions and occasionally on the precipitates. Enzyme assays were also run on uncentrifuged samples.

Absorption experiments were performed in a similar manner; the absorbing antigen was allowed to react with the serum at 5° C. for several hours before the addition of active enzyme. The removal of antibody against the active enzyme by this procedure was considered evidence for the presence of cross-reacting antigens in the absorbing extract. Inhibition values given are based on differences in activity between control serum and antiserum.

Results.—The antitryptophan synthetase activity of whole and fractionated immune serum was determined by measuring the reduction in activity of tryptophan synthetase preparations incubated with control serum and antiserum. From the inhibition values shown in Table 2, it can be seen that immunization with tryptophan synthetase preparations effectively elicits the formation of antibodies against this enzyme.

TABLE 2

INHIBITION OF TRYPTOPHAN SYNTHETASE ACTIVITY BY ANTISERUM, AS A FUNCTION OF TIME

Time at 5° C. (Hours)	Per Cent Inhibition by Antiserum	Time at 5° C. (Hours)	Per Cent Inhibition by Antiserum
0.25	65	48	72
1	66	72	69
24	74	168	53

Two tubes were tested at each time interval, one containing nonimmune (control) serum, and the other, antitryptophan synthetase antiserum. To each tube were added 1.0 ml. of a 1:1 dilution of the appropriate fractionated serum in phosphate buffer (0.1 *M*, pH 7.8), 0.2 ml. enzyme (14 TSU), 0.04 ml. pyridoxal phosphate (40 μgm.), and 0.1 ml. glutathione (5×10^{-3} *M*). At the indicated times the tubes were centrifuged, and the supernatants tested for enzyme activity. Inhibition values are based on difference in activity between control serum and antiserum.

It was of interest to determine two characteristics of this enzyme–anti-enzyme reaction: first, the time course of the reaction and, second, whether the antiserum neutralized the enzyme's activity or merely precipitated the enzyme. From Table 2 it can be seen that the reaction, measured in terms of tryptophan synthetase inhibition, is complete in less than 1 hour. It may be seen from Table 3 that the antiserum neutralizes enzyme activity, since there is no difference in percentage inhibition of enzyme activity between mixtures tested before centrifugation and those tested after centrifugation. Furthermore, no enzyme activity could be detected in the precipitate.

TABLE 3

NEUTRALIZATION OF TRYPTOPHAN SYNTHETASE ACTIVITY BY ANTISERUM

	Per Cent Enzyme Activity Remaining in		
Antiserum	Uncentrifuged Sample	Supernatant Solution after Centrifugation	Precipitate
1	70	74	...
2	52	46	...
3	0	0	0

Determinations were carried out in nonimmune serum and antitryptophan synthetase antiserum, and activity values are based on the difference in activity between these two sera. Antiserum from three rabbits was tested. To each tube were added 1.0 ml. of a 1:1 dilution of the appropriate fractionated serum in phosphate buffer (0.1 *M*, pH 7.8), 0.8 ml. enzyme (14 TSU), 0.04 ml. pyridoxal phosphate (40 μgm.), and 0.1 ml. glutathione (5×10^{-3} *M*). Tubes were stored at 5° C. for 40 hours before assay.

With a constant amount of antiserum and increasing amounts of enzyme, it is possible to standardize a given immune serum in terms of its antitryptophan synthetase activity. Absorption experiments were then carried out with extracts of various mutants to test for the presence in them of proteins antigenically related to tryptophan synthetase.

Extracts of strains td_1 and td_2 were employed in the initial absorption experiments. Aliquots of these extracts were incubated with antiserum of known antitryptophan synthetase titer for 1 hour at 5° C., the mixture centrifuged, and the supernatant solutions tested for their antitryptophan synthetase activity. As can be seen from the data in Table 4, strain td_2 contains proteins sufficiently similar to

TABLE 4

ABSORPTION OF ANTITRYPTOPHAN SYNTHETASE ANTISERUM WITH CRUDE AND FRACTIONATED PREPARATIONS OF STRAINS td_1 AND td_2

Absorbing Antigen	Test Antigen	Per Cent Inhibition of Tryptophan Synthetase Activity by Absorbed Antiserum
None	Tryptophan synthetase	100
td_1—crude extract	Tryptophan synthetase	100
td_1—dialyzed extract	Tryptophan synthetase	100
td_1—fractionated extract	Tryptophan synthetase	100
td_2—crude extract	Tryptophan synthetase	10
td_2—dialyzed extract	Tryptophan synthetase	12
td_2—fractionated extract	Tryptophan synthetase	3

To each tube were added 0.5 ml. of the appropriate fractionated serum, 0.5 ml. absorbing antigen, 0.04 ml. pyridoxal phosphate (40 μgm.), and 0.05 ml. glutathione (2.5×10^{-3} *M*). Tubes were adjusted to equal volume with phosphate buffer (0.1 *M*, pH 7.8). Absorption was continued for 2 hours at 5° C. before the test antigen (0.05 ml. enzyme [7 TSU]) was added. After an additional 30 minutes at 5° C., the tubes were assayed for enzyme activity. Determinations were carried out in nonimmune serum and antitryptophan synthetase antiserum, and activity values are based on difference.

tryptophan synthetase to react with and remove antibody to the enzyme, thereby reducing the anti-enzyme activity of the serum. Furthermore, the same results

are obtained regardless of whether the antiserum-extract mixtures are centrifuged after absorption or not. This observation indicates that the antigenically similar material inactivates or blocks the activity-neutralizing site of the anti-enzyme.

Of particular interest is the finding that extracts of strain td_1 do not contain cross-reacting protein.

Extracts of td_2 were fractionated in a manner similar to that used for purifying tryptophan synthetase preparations, and the final fraction tested for cross-reacting material. It can be seen in Table 4 that the cross-reacting material in extracts of strain td_2 is similar to tryptophan synthetase in its behavior during fractionation. Similar treatment of td_1 extracts failed to yield a preparation containing cross-reacting material.

In view of the differences observed between extracts of strains td_1 and td_2, extracts of other td mutants were examined for antigens which would absorb antitryptophan synthetase antibodies. As may be seen from Table 5, strains td_3, td_6, and td_7 all form proteins which cross-react with antitryptophan synthetase.

TABLE 5

ADSORPTION OF ANTITRYPTOPHAN SYNTHETASE ANTISERUM WITH EXTRACTS OF VARIOUS td MUTANTS AND OF SEVERAL td MUTANTS CARRYING SPECIFIC SUPPRESSOR GENES

Absorbing Antigen	Test Antigen	Per Cent Inhibition of Tryptophan Synthetase Activity by Absorbed Antiserum
td_1su_2—crude extract (0 TSU)	Tryptophan synthetase	100
td_2su_2—crude extract (0.3 TSU)	Tryptophan synthetase	19
td_2su_2—dialyzed extract (0 TSU)	Tryptophan synthetase	0
td_2su_2—fractionated extract (0.4 TSU)	Tryptophan synthetase	4
td_6su_6—crude extract (1 TSU)	Tryptophan synthetase	38
td_6—crude extract (0 TSU)	Tryptophan synthetase	36
td_3su_3—crude extract (0.65 TSU)	Tryptophan synthetase	16
td_3—crude extract (0 TSU)	Tryptophan synthetase	5
td_7—crude extract (0 TSU)	Tryptophan synthetase	13

To each tube were added 0.5 ml. of the appropriate fractionated serum, 0.5 ml. absorbing antigen, 0.04 ml. pyridoxal phosphate (40 μgm.), and 0.1 ml. glutathione (5×10^{-3} *M*). Absorption was continued for 2 hours at 5° C. before the test antigen was added (0.2 ml. enzyme [9 TSU]). After an additional 30 minutes at 5° C., the tubes were assayed for enzyme activity. Determinations were carried out in nonimmune serum and antitrytophan synthetase antiserum, and activity values are based on difference.

Several experiments were carried out with extracts of strains carrying suppressor genes. As may be seen in Table 5, extracts of td_1su_2 (strain td_1 is unaffected by su_2) do not contain cross-reacting proteins. Thus td_1su_2 and td_1 are similar in this respect. It can also be seen in this table that extracts of td_2su_2 *do* remove antibodies to tryptophan synthetase. This is not unexpected, since strain td_2su_2 forms small amounts of tryptophan synthetase.[4] However, what is surprising is that td_2su_2 extracts remove antibody out of proportion to the amount of active tryptophan synthetase they contain. This would suggest that this strain forms the antigen or antigens characteristic of td_2 in addition to the normal enzyme. The results obtained with extracts of strains td_6su_6 and td_3su_3 appear to be similar to those found with td_2su_2. Thus it would appear that suppressed strains possessing suboptimal tryptophan synthetase levels continue to form another protein or proteins, antigenically related to tryptophan synthetase but having no tryptophan synthetase activity.

Discussion.—The various tryptophan requiring strains used in these investigations are noteworthy in that they possess a number of properties in common. All

these strains have an absolute requirement for tryptophan which cannot be satisfied by either indole or anthranilic acid; they have similar requirements for tryptophan; they are characterized by their complete lack of tryptophan synthetase activity; and they all appear to be allelic. In view of these similarities, it may be said that these strains represent mutations of the same or of closely linked interdependent genetic material at the same biochemical locus.[4, 5]

These same mutants, however, do show some very striking differences, particularly in their response to specific suppressor genes;[4, 5] a given suppressor restores tryptophan synthetase formation only in combination with a particular td allele.

The results of the present investigation further demonstrate that differences exist between these allelic strains. Some of the mutants form material closely related antigenically to tryptophan synthetase, but this property is not characteristic of all the alleles. Whether the antigens formed by the various strains differ is not as yet known. It may well be that we are dealing with differences other than the presence or absence of a single cross-reacting antigen. Hence these experiments further strengthen the conclusion that a locus controlling the formation of a specific enzyme can be exceedingly complex and subject to numerous changes. The elucidation of differences between alleles would thus appear to be dependent upon the number of criteria that are applied.

The fact that td_1su_2 does not form cross-reacting material demonstrates that it is the mutant locus and not the suppressor which controls the production of the antigen or antigens. Of particular interest in this regard is the observation that td_2su_2, td_3su_3, and td_6su_6 appear to form both cross-reacting material and tryptophan synthetase. This would suggest that suppressor genes do not completely overcome defects in tryptophan synthetase formation, a conclusion consistent with the fact that suppressed strains form considerably less tryptophan synthetase than the wild-type strain.

One additional point of interest is that all attempts to obtain a suppressor for strain td_1, the strain which does not form cross-reacting material, have been unsuccessful.[4, 5] Td_2 and all the alleles studied except td_7 form cross-reacting material and are suppressed by specific suppressor genes. The suppressibility of strain td_7 has not as yet been demonstrated. These data seem to suggest that the ability to form cross-reacting material and suppressibility may be related. The validity of this correlation must await further study, particularly in cases such as that of td_7.

What is the relationship of the cross-reacting antigen or antigens to tryptophan synthetase? Certainly it is not known at this time, although a great many possibilities exist. The cross-reacting material could be enzyme precursor, altered or incomplete forms of the enzyme, inactivated tryptophan synthetase, or a protein or proteins formed along the same biosynthetic pathway as tryptophan synthetase. Further study is necessary to answer the question.

In any event, the presence or absence of proteins antigenically related to tryptophan synthetase in different members of a group of very similar mutants does suggest that these differences may be a reflection of defects in specific and separate phases of the synthesis of tryptophan synthetase.

Summary.—Preliminary data have been presented on the immunochemical characterization of the tryptophan synthetase–antitryptophan synthetase reaction. Antibody against partially purified tryptophan synthetase preparations from *N. crassa* was found to neutralize enzyme activity. Extracts of several allelic tryptophan requiring mutants, which lack the enzyme tryptophan synthetase, were examined for the presence of proteins which cross-react with antitryptophan synthetase antibody. All but one of the mutants studied were found to possess such material. The cross-reacting material in the mutants behaved as did tryptophan synthetase during the course of purification. Mutants which carry specific suppressor genes, with the attendant formation of low levels of tryptophan synthetase activity, were found to form both active enzyme and cross-reacting material.

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*ON ABELIAN VARIETIES OVER FUNCTION FIELDS**

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In a recent paper[1] on Abelian varieties over function fields, we have shown that to every algebraic system of Abelian varieties, defined over a field K, can be assigned two invariants, called the *K-image* and the *K-trace*. Let $K(u)$ be a primary extension of K, and let A^* be an Abelian variety defined over $K(u)$; then the K-image A of A^* over $K(u)$ is an Abelian variety over K characterized by the existence of a rational homomorphism F (called the *canonical homomorphism*) of A^* onto