STUDIES OF THE MULTIPLE FORMS OF TYROSINASE IN *NEUROSPORA CRASSA* AND THEIR RELATIONSHIP TO GENETIC DIFFERENCES IN THERMOSTABILITY

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ABSTRACT

STUDIES OF THE MULTIPLE FORMS OF TYROSINASE IN NEUROSPORA CRASSA AND THEIR RELATIONSHIP TO GENETIC DIFFERENCES IN THERMOSTABILITY

by Morton S. Fuchs

Two strains of Neurospora Crassa, differing with respect to a pair of alleles (T^S and T^L), produce thermostable and thermolabile tyrosinase respectively. Fractionation of the enzyme from each into three components has been accomplished by means of continuous-flow paper electrophoresis and ion-exchange chromatography. A conversion phenomenon among these components is observed in extracts of both strains.

Thermal inactivation studies show that the difference in thermostability of the enzyme from the two strains is not attributable to differences among and conversion of the three components in each.

Gel-filtration and sucrose gradient centrifugation show that the three components produced by each strain do not differ significantly in molecular weight, and that the tyrosinase of the two strains also does not differ in molecular weight. This is interpreted to mean that differences in aggregation are not responsible for the differences among the three components in each strain, and that

the difference between strains in heat stability cannot be attributable to differences in molecular weight.

It seems most probable that the three tyronsinase components produced by each strain differ in tertiary configuration. The structural basis of the difference between strains remains unelucidated. None of this evidence excludes a difference in tertiary structure, but differences in primary structure await verification.

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Thanks are extended to Mr. Sheldon D. Parzen for his interest and encouragement and to all other members of the "Fox group" for creating a stimulating environment.

Dedicated to my wife Mary ann and

to my wonderful Mother and Father

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INTRODUCTION

One of the fundamental concepts of modern biology has been the so-called "central dogma" (1), which may be stated as follows: There is a DNA code which specifies an RNA code which in turn determines the amino acid sequence of a particular protein (2). This concept can be diagrammed in the following way:

DNA $\xrightarrow{\text{Transcription}}$ RNA $\xrightarrow{\text{Translation}}$ protein (enzyme) $\dashrightarrow$ products (3). In the above, the arrows are presumed to indicate a flow of information (transcription and translation), and the last arrow is meant to signify the products of the enzyme activity.

Implicit in this concept is the prediction that the information contained within the amino acid sequence of a polypeptide chain is sufficient to cause it to fold into the precise configuration required for its particular function (4). According to this hypothesis, the polypeptide chain will take the three-dimensional configuration with the lowest configurational free energy, and hence under any specific set of conditions will be the one that is thermodynamically the most stable (5). As pointed out by Anfinsen et al (5), this 'thermodynamic' hypothesis has the great virtue (besides its innate simplicity) of being amenable to test. A model experiment would be to convert a native protein into a linear polypeptide chain devoid of disulfide

bonds and of secondary and tertiary structure and then to restore this unfolded and reduced ("denatured") protein to its original configuration without the introduction of additional "information" (5). Studies of just this sort have been carried out on a number of different proteins, some of which contain disulfide bonds and others that do not. Of the disulfide bond containing proteins studied, bovine pancreatic RNase (6, 7), egg white lysozyme (8, 9), tak-amylase A (10), bovine trypsin (11, 12), Escherichia coli alkaline phosphatase (13), swine pepsinogen (5), human serum albumin (14), and soybean trypsin inhibitor (15) all show reversible denaturation (i.e. unfolding followed by proper refolding). Successful experiments of this type have also been performed with such diverse proteins as bacterial α-amylase (16), yeast enolase (17) and bacteriophage lysozyme (18, 19) all of which do not contain disulfide bonds.

The criteria used to determine the 'nativeness' of the renatured proteins mentioned has included the following comparisons with native proteins: specific activity, optical rotation, viscosity, ultra-violet spectrum, antigenic identity, peptide maps, chromatography, crystal form, sedimentation, and electrophoretic properties. These criteria were used in various combinations for the different proteins mentioned. In all cases where estimates

could be made for random yields, i.e. the probability of purely random sulfhydryl pairings to form the same disulfide bonds that are present in the native protein (5, 20), the actual recovery of activity (using combinations of the above criteria) was always considerably above that of the calculated random recovery.

In spite of the preponderance of indirect evidence supporting Crick's statement that "folding is simply a function of the order of the amino acids" (1) unequivocable evidence exists for only one protein, RNase. This was produced by White (6) when he showed that the X-ray diffraction pattern of reoxidized RNase was indistinguishable from the native enzyme. Indeed, the generality of the phenomenon of spontaneous folding may be questioned since as pointed out by Helinski and Yanofsky (21), "there have been many unsuccessful attempts to restore the tertiary structure or activity of other proteins denatured under relatively mild conditions".

A serious challenge to the "thermodynamic" hypothesis arises from the work of Berson and Yalow (22, 23). They found that certain antisera can distinguish between sperm whale and pork insulins, proteins identical in amino acid sequence. They concluded, "it would seem that, in addition to amino acid sequence, the precise configuration of folding of the protein molecule is determined by the genetic appartus". Conversely, the tertiary structures of the α- and β-chains of horse hemoglobins (24) and sperm whale myoglobin

(25) have been worked out, and there exists a close similarity between these two proteins in their tertiary structures, despite marked differences in their amino acid compositions. In addition, H.C. Watson and Kendrew compared amino acid sequences of human hemoglobin and sperm whale myoglobin and found remarkably few correspondences, again despite their similar tertiary structures (26).

Another complicating feature of the "thermodynamic" argument of spontaneous folding, is the discovery and purification by Anfinsen and coworkers (27) of an enzyme isolated from beef liver which catalyzes the 'renaturation' of RNase, egg white lysozyme, and soybean trypsin inhibitor. Previous work on the reactivation of reduced RNase (7) had shown that air oxidation of the polypeptide chain of the reduced enzyme results in a protein with native conformation and full activity. Such oxidation occurs spontaneously, but relatively slowly. By choosing the lowest protein concentration technically feasible (0.01 mg/ml), a relatively high pH (8.2), and the optimal temperature (24°C) the lowest half-time reactivation achieved by air oxidation was 20 minutes (28). Reactivation in the presence of the enzyme, however, proceeds rapidly under more physiological conditions, i.e. pH 7.0 to 7.4 and 37°C. With partially purified enzyme under these conditions half-maximal reactivation occured in less than 3 minutes (29) and in the purest

preparations obtained, "the rate is in the range that would be expected for an enzyme which catalyzes the proper folding of a newly synthesized polypeptide chain judged by estimates for the rate of protein synthesis in mammalian cells" (27). It is clear from the above studies that spontaneous folding does not constitute a major mechanism for the determination of tertiary structure of RNase, egg white lysozyme or soybean trypsin inhibitor (17, 29).

In recent years it has become evident that a great number of proteins are composed of subunits which are able to associate and dissociate under proper experimental conditions (30). In fact, Changeux (31) points out that all known regulatory enzymes are multimers. The ubiquitousness of protein subunit structure indicates an important biological function, and indeed this subject has recently been given extensive discussion (32, 33). Two major ideas, not mutually exclusive, have emerged.

Monod et al (34-36) propose that specific regulatory interactions of enzymes generally involve topographically distinct stero-specific sites and are mediated through a conformational alteration (allosteric effects). An allosteric enzyme is defined as one which can combine with a substance sterically unrelated to the substrate at a site different from the active site and, as a result, undergo a conformational change or allosteric transition. According to Changeux (35) "the ability to mediate allosteric interactions appears

to be directly dependent on the presence of a quaternary structure".

The second idea concerning the biological importance of subunit interactions derives from the extensive work done on tryptophan synthetase of Escherichia coli by Yanofsky and his group (37). The finding that the β protein subunit of the E. coli tryptophan synthetase has serine deaminase activity and that this activity disappeared when the β protein was combined with the α protein subunit (38), led D.M. Bonner to postulate that protein subunit interactions have great evolutionary significance. Thus, Bonner's evolutionary scheme suggested (as stated by T. Crighton, 39), "that the combination of two enzyme molecules to form an enzyme complex which can efficiently combine two previously independent enzymatic activities may be the basis for the subunit structure of some enzymes". The fact that "hybrid" enzymes appear in heterozygotes, as in the cases of the esterases in maize (40), the acid phosphatases in Tetrahymena (41), and the alkaline phosphatases in Drosophila (42) among others, caused Fincham (33) to speculate along similar lines. He points out that there is no evidence that the hybrid enzymes thus formed are functionally superior to 'pure' protein types but states: "It seems, nevertheless, reasonable to suggest that hybrid proteins are sometimes of advantage to the organism carrying them". If so, according to Fincham, this would form part of the explanation of heterosis and give the hybrid a

distinct selective advantage which through evolution would bring about a state of 'permanent hybridity'.

Whatever the importance of the multimeric state of many proteins the question of the mechanism of their genetic control becomes pertinent. The original 'dogma' concerned itself with primary and tertiary structure. The more modern version incorporates the idea of subunit structure and as expressed by Helinski and Yanofsky states (21): "The specific and unique association of the subunits of an enzyme can also be considered to be directed by amino acid sequences and thus by the conformation of the polypeptide chain, rather than by the control or direction of a specific cytoplasmic structure". The evidence for spontaneous formation of quaternary structure comes from many sources and has been demonstrated in both in vivo and in vitro systems (43-50). An outstanding case in point is lactic dehydrogenase of birds and mammals. In a wide range of species this enzyme has been shown to occur in five isozymic forms. Kaplan and his group (51) have shown that the active form of the enzyme is a tetramer of two interchangeable electrophoretically distinct kinds of polypeptide chains. One kind, H, predominates in heart muscle and the other, M, in skeletal muscle. The pure tetramers are designated H4 or M4 as the case may be, and the heterotetramers as H3M, H2M2, and HM3 respectively. A binomial distribution of the five forms

is found when the two types of enzymes are synthesized by one cell type; the distribution depends on the number of H and M subunits present.

Evidence of the sort presented above coupled with the fact that efforts to demonstrate specific binding mutations involving enzyme subunits have failed, argue very strongly in favor of the spontaneous synthesis of quaternary structure. However, as in the case of tertiary structure, there are complications. Thus, Zipser and Perrin (52) observed that the aggregation of β-galactosidase from subunits to make active enzyme proceeds at a much faster rate in the presence of ribosomes. Other types of protein-protein interactions other than enzyme subunits exist and are thought to be under genetic control. Edgar and Wood (53) have implicated direct genetic control of protein-protein binding in their complementation studies with phage amber mutants. Green and Hechter (54) in a paper concerned with the assembly of membrane subunits in mitochondria conclude with this statement: "Our consideration of the assembly in membranes has served to remind us that the heredity apparatus contains coded information, not only for the sequences of amino acids in proteins, but also for the sequences of proteins in macromolecular assemblies".

The argument that tertiary and quaternary structures of enzymes are products of the primary structure of their subunit

components is an attractive one, but not necessarily correct in all cases. There are enough indications in the literature to warrant further investigations into the possibilities that other mechanisms controlling tertiary and/or quaternary structure do indeed exist.

The enzyme tyrosinase (EC 1.10.3.1) in Neurospora crassa promised to be an ideal system for the uncovering of additional genetic control of tertiary and/or quaternary structure, superimposed upon the known mechanisms controlling primary structure. Extensive studies of Neurospora tyrosinase have been carried out over the last fifteen years. Most of the data accumulated over this period have come from the respective laboratories of A.S. Fox and N.H. Horowitz. Differences in results obtained by these two workers have led each to contradictory interpretations of the mechanisms of genetic control involved in this system. Genetic studies and detailed analyses by Horowitz and co-workers have revealed the following: Working with wild strain 4A of Neurospora crassa, Horowitz and Fling reported that two alleles at a locus designated T determine different forms of tyrosinase. The two enzymes are distinguished by a striking difference in stability at 59°C. and are therefore designated thermostable and thermolabile. The thermal difference was found to be simply inherited, the stable, S, form of the enzyme being determined by a gene T^S and the labile, L, form by its allele T^L, located on the mating type chromosome (55, 56).

Only one form, S or L is found in a homocaryon, depending on the T allele present (55). In heterocaryons possessing both the T^S and T^L genes both forms of the enzyme are demonstrable (57). Crude preparations of the L enzyme exhibit a half-life at 59° of 3-4 minutes while the S form at the same temperature had a half-life of 30 minutes. Crystalized L tyrosinase has a half-life of 5 minutes as opposed to the crystalline S form which exhibits a half-life at 59° of 65 minutes. The kinetics of inactivation is first order for both (58). The energies of activation for the thermal degradation of the enzyme are also different. Sueoka (59) reports L tyrosinase has an energy of activation of heat inactivation of 84,000 calories per mole between 53°-59° and 59,000 calories per mole between 49°-53°. For S tyrosinase, μ = 90,000 calories per mole between 61°-67° and 61,000 calories per mole between 57°-61°. Both forms of the enzyme exhibited considerable variability in their stability at a variety of temperatures both in crude and purified preparations. The two enzymes can also be distinguished by their different electrophoretic mobilities by paper strip ionophoresis (60). At pH 6.0 both forms migrate toward the cathode with the L form being fastest (61).

A detailed study by Sussman (61) of thirty-three substrates, which included mono-, di-, tri-, and conjugated phenols, revealed no difference in specificity between L tyrosinase and S tyrosinase.

Michaelis-Menten constants for the two forms of enzyme acting on L-tyrosine, and L- and D-dihydroxyphenylalanine (dopa) were also found to be similar, as were their pH optima and their response to several inhibitors. On the other hand, the activity of the thermolabile enzyme was lost more rapidly than that of the thermostable one after incubation in high concentrations of urea or formamide. Sussman interprets this data to indicate that the difference between the two forms of tyrosinase reside in their secondary and tertiary structures. Crystalline L and S forms show no difference in their amino acid compositions, turnover number or "fingerprint" patterns (58). It is interesting to note that neither tyrosinase contains cystine and therefore neither forms disulfide bonds (58). Two other forms of tyrosinase were found in Neurospora by Horowitz et al (61). These were designated Puerto Rico-15 (or PR-15) and Singapore-2 (or Sing-2). Both the PR-15 and Sing-2 tyrosinases segregate with either L or S. They are distinguishable from one another on the basis of heat stability and/or electrophoretic mobility. Horowitz and coworkers (62) also reported the presence of two other loci, ty-1 and ty-2 not linked to each other which are epistatic to the T locus. The presence of genes ty-1 and/or ty-2 (as opposed to ty-1^+ and ty-2^+) results in the cessation of tyrosinase production. The effect of the ty genes can be overcome by starving the mycelia or by adding certain D-aromatic amino acids to the growth medium.

The form of the induced enzyme is dependent upon which allele of the T locus (T^S, T^L, T^{PR-15} or T^{Sing-2}) is present. Horowitz has concluded that the T locus is a structural one and that the ty-1 and ty-2 loci are regulatory. He attributes the different forms of tyrosinase to genetic alteration at the T locus resulting in a change in primary structure thereby accounting for the differences in heat stability and electrophoretic mobility between the tyrosinases (63).

Early data obtained by Fox's group (64, 65) working with the albino-2 mutant 15300, indicated that tyrosinase concentration and specificity in Neurospora are controlled by two different genetic systems. Later Fox et al (66, 67) reported the existence of different forms of the enzyme utilizing a continuour flow paper electrophoresis technique. The crude tyrosinase extracts separated into three components T_1, T_2, and T_3, two or three of which were present in any one thallus. In order to rule out heterocaryosis as the reason for the simultaneous appearance of the multiple forms, hyphal tip and ascospore isolates were examined for their tyrosinase content. The results clearly indicated that homocaryotic cultures are capable of producing two or three forms of the enzyme simultaneously (68). Further analysis of T_1, T_2, and T_3 led to the following observations (66-68): The activation energy for the thermal degradation of T_1, 95,800 calories per mole, was similar to Horowitz's L form (84,000 calories per mole) and for T_2 and T_3,

μ = 60, 900 and 57, 300 calories per mole respectively, virtually identical to the S form (64, 000 calories per mole). There were no differences in the Michaelis constants of the three enzymes T_1, T_2 and T_3 with L-tyrosine and L-dopa as substrates, nor were there any differences with regard to their dopa/tyrosine activity ratios. In material that contained all three forms, T_1, T_2, and T_3 constituted 5.9, 12.8 and 81.3 percent respectively of the total tyrosinase in fresh extracts. After incubation at 5° C. for six days of an aliquot of the same fresh extract T_3 had increased to 91.6 percent of the total. The gain in T_3 was exactly accounted for by concomittant loss of T_1 and T_2, with no change in overall activity of the incubated aliquot. A linear scheme of interconversion was suggested when an electrophoretic analysis of 33 ascospore isolates showed that T_1 is found only in conjunction with T_2 and T_3, while T_2 and T_3 may appear together but only T_2 appears alone. Based on his own observations, that of Horowitz's group and the fact that a tyrosinaseless mutant which segregates with the T locus has never been found, Fox suggested that the T locus controls the equilibrium in the interconversion of the alternate forms of the enzyme, and that the difference between the forms is not in their primary structure but in their tertiary structure (67, 68). Thus, this interpretation would mean the T locus determines the tertiary structure of tyrosine and that another, as yet unidentified, locus determines

primary structure.

The purpose of the present work is to determine which of these two interpretations is correct and/or if a reconciliation of the two points of view is possible. Since both groups had been using different methods of growth and extraction as well as different stocks the first obvious step was to minimize these differences. To this end all the reported data heretofore was obtained with Horowitz's T^L and T^S stocks, and his methods of growth and extraction were adhered to closely.

MATERIALS AND METHODS

Strains of Neurospora crassa producing either the S or the L form of tyrosinase were used throughout this study. The T^S strain (No. 69-1113a) was kindly supplied by Dr. Norman H. Horowitz of the California Institute of Technology, and the T^L strain (No. 320) was obtained from the Fungal Genetic Center located at Dartmouth College in New Hampshire. They were maintained on solid Westergaard and Mitchell minimal medium (69).

In order to obtain as much enzyme as possible three methods of growth and induction were used, duplicating the procedures reported by Horowitz et al (70, 58, 71). The first consisted of growing cultures in 125 ml. erlenmeyer flasks containing 20 ml. of Vogels N liquid minimal medium (72) at 25° C. After two days of growth the medium was decanted and the mycelial mat was washed twice with 20 ml. of sterile distilled water. The washing consisted of gently swirling the mycelium around and then decanting the water. After the second water wash, 5 ml. of sterile sodium phosphate buffer, 0.02 M, pH 6.0, was added to each flask. The flasks were incubated in the dark at 25° C for 48 hours. At the end of this time the mycelial mats were collected by filtration, washed thoroughly and quickly with cold water, squeezed dry and stored at -25° C. until needed. In the second method N. crassa was

grown in carboys. A two gallon carboy containing six liters of Vogels N liquid medium was innoculated with a condial suspension and allowed to grow with aeration in the dark at 25° C. for 3-4 days. The medium was aseptically replaced with sterile 0.05M, pH 5.5 sodium phosphate buffer, placed in the dark, and aerated for an additional 4-5 days at 25° C. before it was harvested. After harvesting the mycelium was washed as before and frozen until needed. The last procedure, and the one that gave the highest yield of enzyme activity, consisted of growing the fungus in 6 liters of half strength Vogel's medium N containing 1/2 percent sucrose in the dark, at 25° C., with aeration for 72 hours. At the end of this time one gram of D-phenylalanine was added to the growing culture and the carboy was allowed to sit in the incubator without changing conditions for another 4-5 days before harvesting. As before, the mycelium was thoroughly washed in cold water and stored at -25° C. until needed. This technique yielded 30-60 grams wet weight of mycelium per carboy.

The frozen mycelium was ground into small pieces with a mortar and pestle. Ice cold buffer was added to a concentration of 2 ml. buffer to 1 gram of mycelium. The resulting suspension was then homogenized in an all-glass Duall tissue grinding apparatus in an ice bath for five minutes at 500 rpm. When more than 20 ml. of buffer was needed the suspension was homogenized in a pre-cooled

Waring blendor operated at 4° C. at high speed for one minute. The homogenate was centrifuged at 37, 500 xg for 20 minutes at 4° C. and the precipitate was discarded. The clear supernatant constituted the crude extract. In some experiments an aliquot of the homogenate was used immediately while the rest was placed in the cold with stirring for varying periods of time before centrifugation.

Enzyme activity was determined by spectrophotometric measurement, at 475 mμ, of the rate of dopachrome formation resulting from the oxidation of L-dihydroxyphenylalanine (L-dopa) (73). Reactions were carried out in spectrophotometric cells at a controlled temperature of 25° C., regulated by a Haake circulating water bath. Reactions mixtures consisted of 1.0 ml. of appropriate enzyme dilution and 2.5 ml. of 0.1 M sodium phosphate buffer, pH 6.0, containing 3.6 mg. of L-dopa. Absorbancy measurements were automatically recorded at 3 second intervals with a model 2000 Gilford recording spectrophotometer.

In spite of the recommendation of the Enzyme Commission of the International Union of Biochemistry that an enzyme unit be defined as the amount of enzyme which catalyzes the transformation of one mole of substrate per minute under defined conditions (74) the tyrosinase unit reported here will be the one used by Fox and Burnett (67). Their unit is defined as that amount of enzyme which results in an initial rate of change of absorbance at 475 mμ of

10^{-3}/min./cm. light path. Taking into consideration that this assay measures the rate of dopachrome appearance rather than the disappearance of dopa and that 2 moles of dopa are ozidized for every mole of dopachrome produced (58), and given the molar extinction coefficient of dopachrome at 475 mμ of 3600 (75), then in a total volume of 3.5 ml. with L-dopa as substrate 514 of these units equal one Enzyme Commission Unit. Since our spectrophotometric assay gives us a resolution of 0.001 optical density (O.D.) per minute the unit used should be sensitive enough to reflect this resolution. The Enzyme Commission Unit in this context would obscure real differences in enzyme activity by compressing the numerical range of activities actually found in this study.

Electrophoretic fractionation was accomplished by continuous-flow paper electrophoresis on Whatman No. 31ET filter paper, 35.5 x 37.5 cm., in a Spinco Model-CP cell. Details of this procedure have previously been published by Fox and Burnett (67).

For the determination of thermostabilities the pH of the enzyme fractions were adjusted to pH 6.0 in 0.1 M sodium phosphate buffer either by dialysis or by passage through a G-25 Sephadex column. At the beginning of each experiment, the sample being tested was brought rapidly from room temperature to that of a constant-temperature water bath ($\pm$ 0.25°). At predetermined intervals, aliquots were pipetted into tubes immersed in an ice

bath. These were then brought up to 25° C. and assayed for enzyme activity.

Protein concentration was determined by the method of Lowry et al (76), using bovine serum albumin as a standard or by the absorbance obtained at 280 and 260 mμ respectively using Kalckar's formula (77).

The experimental procedures outlined in detail by Martin and Ames (78) for the handling of sucrose gradients were followed closely. Only linear gradients were used and were of 5 to 20 percent sucrose made up in various buffers as indicated, to a volume of 5.3 ml. The volume of enzyme preparation applied to the gradient was 0.2 ml. A standard was always included in a separate tube in every experiment. The standard used was 0.2 ml. (2mg.) of twice crystallized bovine hemoglobin (sigma Chemical Company) and was assayed by absorbancy at 410 mμ. After centrifugation for 20-24 hours at 34,000 rpm and 4° C. in a SW-39 rotor in a refrigerated model L-2 Spinco centrifuge, 10 drop fractions were collected by a device similar to that described by Martin and Ames (78). Each tube yielded an average of 400 drops or approximately 0.14 ml. per fraction. Of this amount, 0.10 ml. was routinely assayed for enzyme activity.

Ion exchange chromatography was performed using the anion exchanger diethylaminoethylcellulose (DEAE-Cellulose),

obtained from Carl Schleicher and Scheull Company, New Hampshire. Cleaned (79) DEAE-cellulose was repeatedly sifted through a No. 100 standard sieve (149 microns) while suspended in distilled water, to remove the fines. After drying, the DEAE-cellulose was resuspended in 0.01 M, pH 7.2 sodium phosphate buffer and the slurry was poured into a column. At least three volumes of this buffer was passed through the column before a sample was applied. The enzyme preparation to be fractionated was in 0.01 M, pH 8.0-8.2 sodium phosphate buffer and elution of the enzyme was accomplished using 0.01 M concentration of this buffer with increasing amounts of sodium chloride, either in step-wise fashion or with a linear gradient (80). The size of the columns used was 12 x 400 mm or 25 x 900 mm and the flow rates obtained with gravity were between 0.5 ml. per minute to 1.5 ml. per minute. All fractionation was carried out at 4° C.

RESULTS

A. ASSAY METHODS

Figure 1A shows the results of a typical assay using undialyzed extract. The lag periods and the discrepancies in activities between the various dilutions were exhibited by every extract, T^S or T^L, regardless of the method of growth. When these extracts were dialyzed overnight against the appropriate buffer, or when buffer exchange was accomplished by gel filtration using G-25 Sephadex, the lags and the dilution discrepancies were eliminated (Figure 1B). These results suggest that a small molecular weight tyrosinase inhibitor was present in the extracts. Horowitz and Shen (81) reported the presence of an inhibitor of tyrosinase in Neurospora crassa which was lost after dialysis. They also demonstrated that when the fungus was grown on a low sulfate-containing medium (0.00785 gm. of $MgSO_4 \cdot 7H\ 0$ + 0.4 $MgCl_2 \cdot 6H\ 0$ per liter, instead of 0.5 gm. of $MgSO_4 \cdot 7H\ 0$) the inhibitor could not be detected. Growth on low sulfate medium was tried but did not result in elimination of the apparent inhibitor in the extracts.

When undialyzed T^L or T^S extracts were incubated for long periods in the cold there was a gradual loss of the inhibitor effect. However, this observation was not consistent and varied qualitatively and quantitatively from extract to extract.

Figure 1A. Typical assay of T^S at various dilutions before dialysis or passage over G-25 Sephadex.

Amount Assayed (ml)	Observed Activity (Units)		Activity/ml (Units)	
	1st Minute	Maximum	1st Minute	Maximum
1.0	110	220	110	220
0.5	140	240	280	480
0.1	35	75	350	750

Figure 1B. Typical assay of T^S at various dilutions after passage over G-25 Sephadex.

Amount Assayed (ml)	Observed Activity (Units)		Activity/ml (Units)	
	1st Minute	Maximum	1st Minute	Maximum
1.0	760	760	760	760
0.5	325	325	750	750
0.1	75	75	750	750

FIGURE 1A. Typical assay of T^S at various dilutions before dialysis or gel-filtration through G-25 Sephadex. Absorbancy calculated for 1.0 ml abstract in each case.

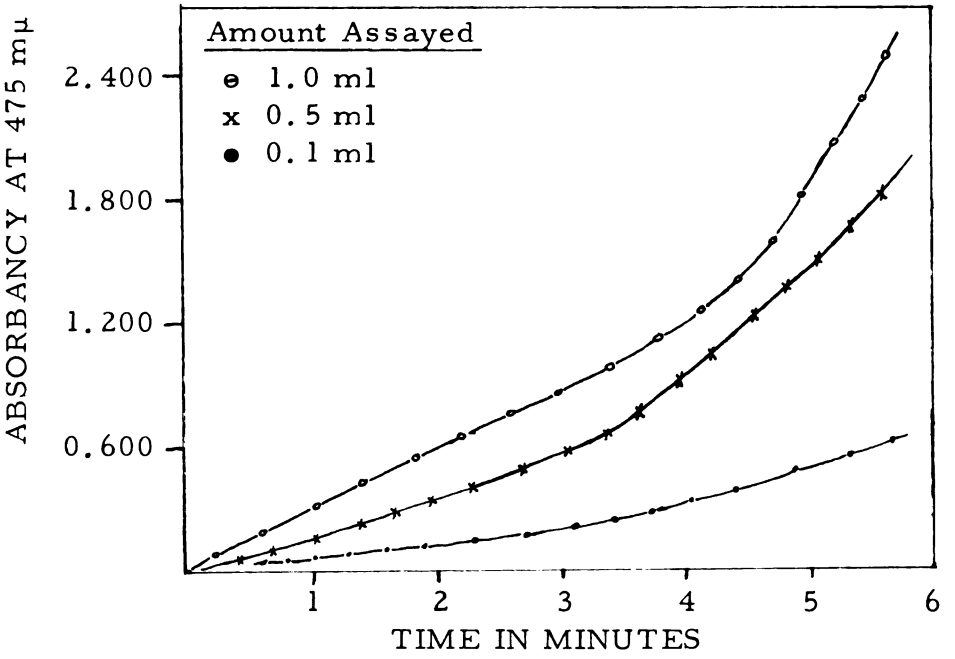
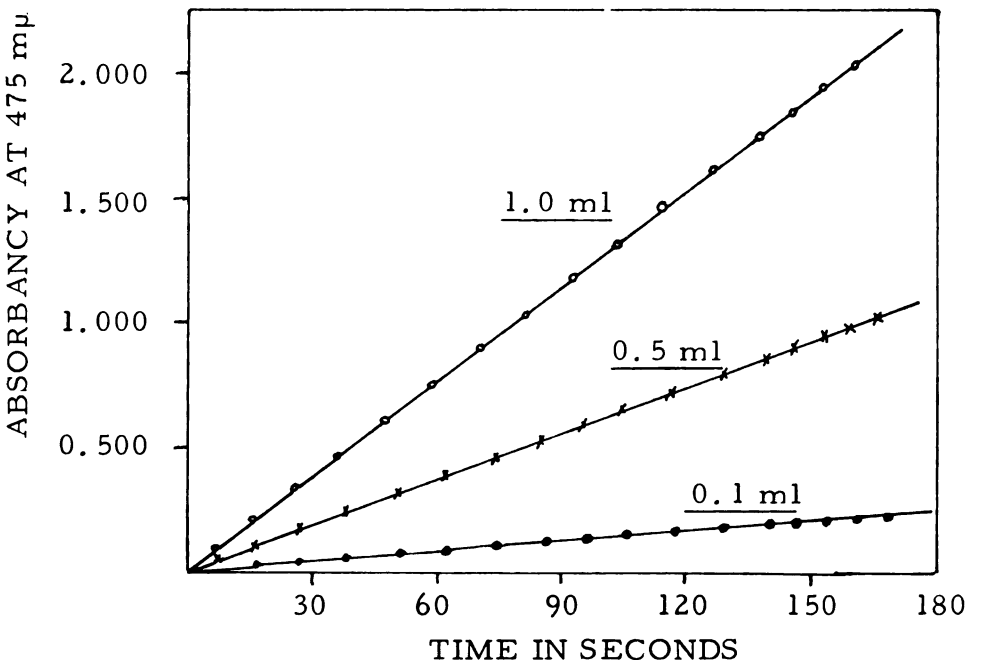


FIGURE 1B. Typical assay of T^S at various dilutions after passage over G-25 Sephadex.



In order to determine the true activity of any extract, dilutions were made until a constant activity per ml was obtained. To ascertain if this was the true activity the extract was dialyzed and then assayed again. In all extracts tested this way, the activity per ml obtained by dilution equaled the activity per ml of the same dialyzed extract. The reliability of the dilution method to obtain the true activity was verified in over 100 different extracts.

No trace of the inhibitor effect was apparent in any of the fractions of tyrosinase obtained after continuous-flow electrophoresis, ion-exchange column chromatography on DEAE cellulose, or sucrose gradient centrifugation.

B. ANALYSIS BY CONTINUOUS-FLOW ELECTROPHORESIS

When crude, undialyzed T^L and T^S extracts are subjected to continuous-flow electrophoresis and the samples collected at the bottom sheet are analyzed for enzyme content, it is evident that more than one tyrosinase component exists for each. Moreover it is clear that their electrophoretic patterns are different (Figures 2 and 3). Adopting Fox and Burnett's nomenclature (67), the most cathodic fraction, tubes 3-9, will be called T^L3 or T^S3; tubes 10-15 will be called T^L2 or T^S2; and tubes 16-25 will be referred to as T^L1 or T^S1.

The T^L extracts showed much more variability in their

FIGURE 2. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet.

Sample: Crude, undialyzed T^L extract.

Sample Volume: 75 ml

Total Activity: 52, 500 units.

Activity Recovered: 25, 426 units (48.4°/0)

Conditions of Electrophoresis:

Time: 60 hours

Buffer: sodium barbital, pH 8.6, I = 0.02

Constant voltage (960 volts) with current of 40-50 milliamperes.

FIGURE 2. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet; T^L.

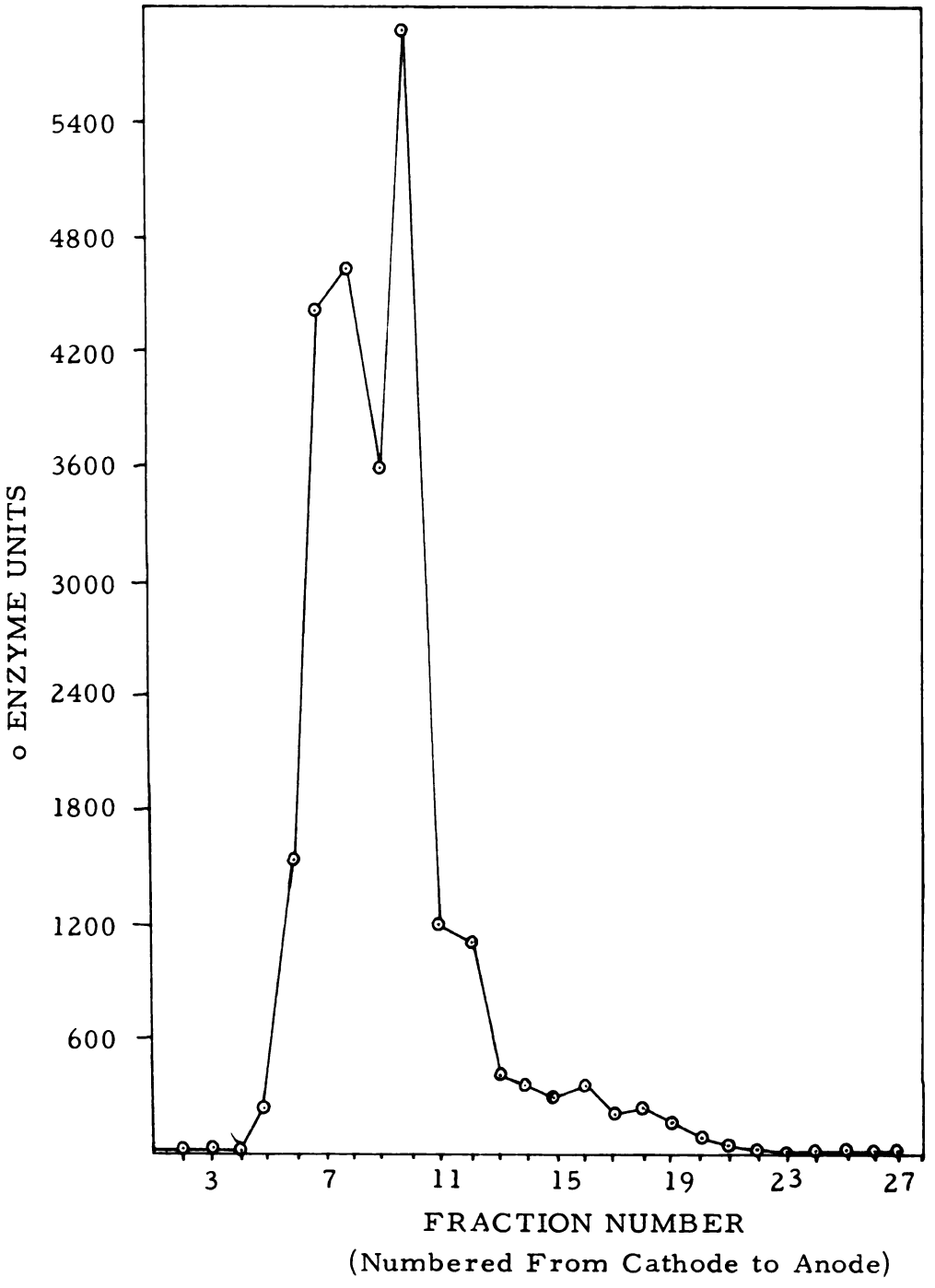


FIGURE 3. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet.

Sample: Crude, undialyzed T^S extract.

Sample Volume: 20 ml

Total Activity: 100,000 units

Activity Recovered: 49,655 units (49.7°/o)

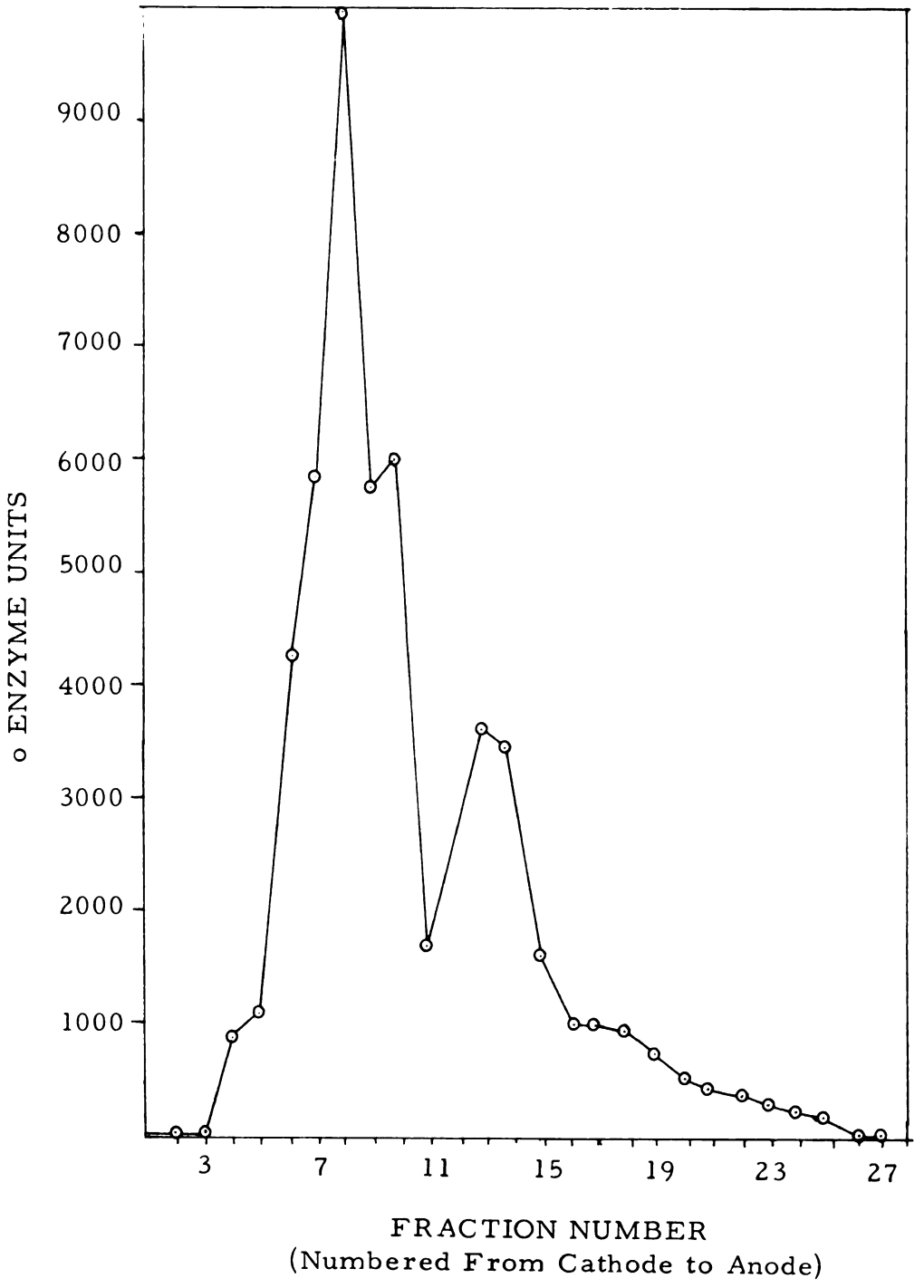
Conditions of Electrophoresis:

Time: 18 hours

Buffer: sodium barbital, pH 8.6, I = 0.02

Constant voltage (960 volts) with current of 40-50 milliamperes.

FIGURE 3. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet; T^S.



electrophoretic patterns (compare Figures 2, 4, and 6A) than T^S. However, the ratio of $T^L2:T^L3$ was always greater than $T^S2:T^S3$.

Dialysis of crude T^L or T^S extracts against pH 8.6 barbital buffer, and subsequent continuous-flow electrophoresis, reveals a shift in the direction of T2 or T3. Figure 4 shows one such experiment. The T^L extract used in this case was almost all T^L2 before dialysis. After dialysis it was converted almost entirely to T^L3. These results parallel those obtained by Fox and Burnett (67) using their 131a-15300 strain of Neurospora crassa.

In order to test the possibility that the conversion of T^L2 to T^L3 was due to change in molecular weight, gel filtration column chromatography on G-100 Sephadex was performed. Dialyzed and undialyzed T^L extracts were chromatographed separately and the fractions collected were analyzed for enzyme content. The extracts were similar, exhibiting a single species of identical chromatographic mobility, and presumably of identical molecular weight. The conversion of T^L2 to T^L3, therefore, is not attributable to changes in molecular weight caused by dialysis.

In contrast, the electrophoretic components do not exhibit conversion after isolation. In an experiment to determine the stability of the electrophoretic components, T^L3 and T^L2 (tubes 3-8 and 10-15, respectively, from Figure 6A) were collected. They were concentrated by dialysis against 25 percent Ficoll (Pharmacia

FIGURE 4. Analysis of fractions collected from two separate continuous-flow electrophoresis sheets.

Sample: Crude undialyzed T^L extract
Crude dialyzed T^L extract

Sample Volume: Undialyzed = 18 ml
Dialyzed = 30 ml

Total Activity: Undialyzed = 7,200 units
Dialyzed = 8,700 units

Activity Recovered: Undialyzed = 6,208 units (86.2%)
Dialyzed = 1,824 units (20.9%)

Time of Electrophoresis: Undialyzed = 20 hours
Dialyzed = 25 hours

FIGURE 4. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet; T^L

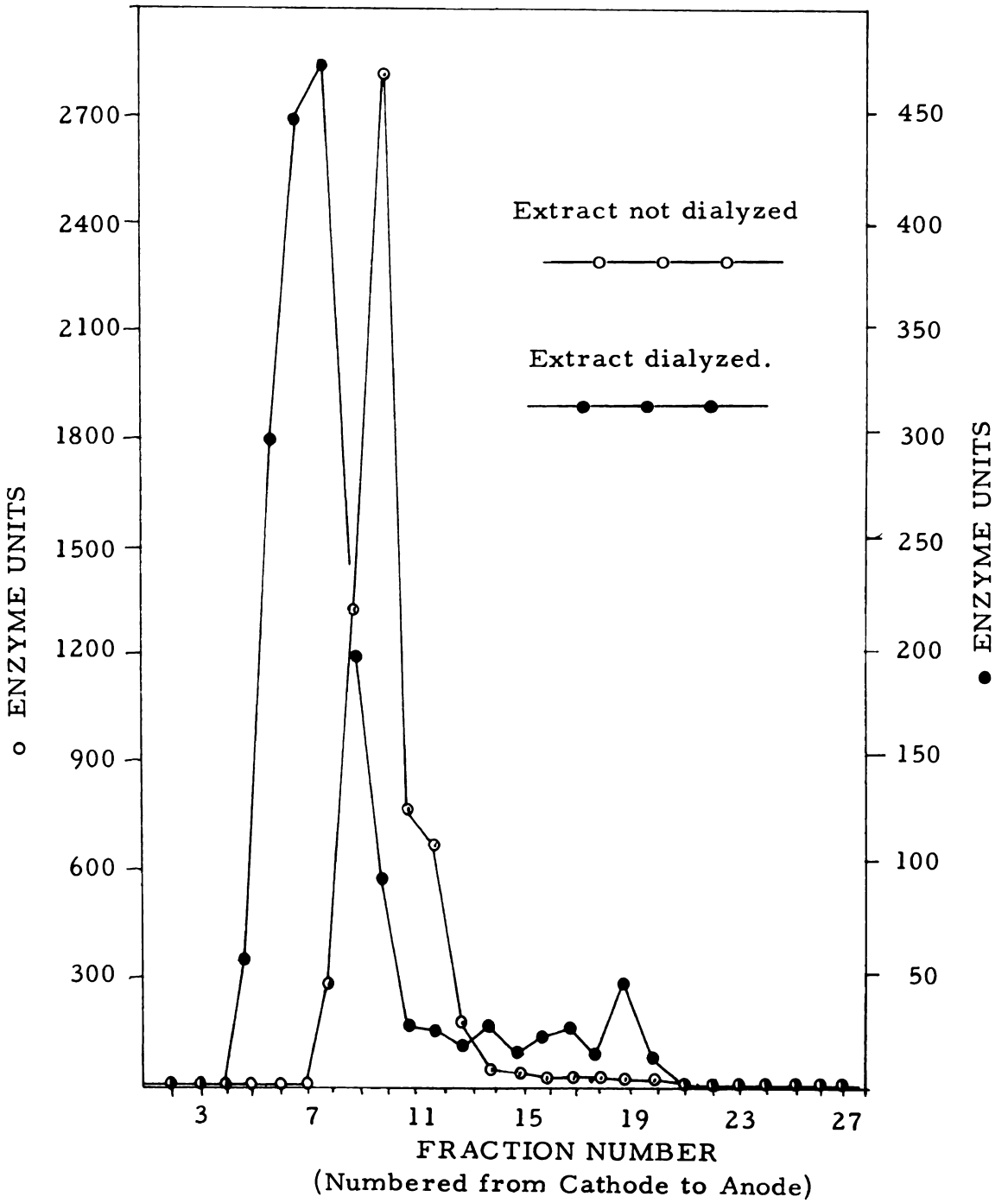


FIGURE 5A. Analysis of fractions collected after gel filtration chromatography of undialyzed, crude T^L extract on G-100 Sephadex.

Dimensions of Column Bed: 1.8 cm by 22.5 cm

Equilibrated and eluted with sodium barbital buffer, pH 8.6, I = 0.02.

Sample: 5 ml of undialyzed, crude T^L extract, in barbital buffer.

Total Activity Applied to Column = 3,250 units

Total Activity Recovered From Column = 3,250 (100%)

Fraction Size = 5 ml.

FIGURE 5B. Analysis of fractions collected after gel filtration chromatography of dialyzed, crude T^L extract on G-100 Sephadex.

Dimensions of Column Bed: 1.8 cm by 22.5 cm.

Equilibrated and eluted with sodium barbital buffer, pH 8.6, I = 0.02.

Sample: 5 ml of dialyzed, crude T^L extract. Dialysis was carried out in cold overnight against barbital buffer.

Total Activity Applied to Column = 2,750 units

Total Activity Recovered From Column = 2,700 units (98.2%)

Fraction Size = 5 ml.

FIGURE 5A. Gel filtration column chromatography of undialyzed crude T^L extract in G-100 Sephadex.

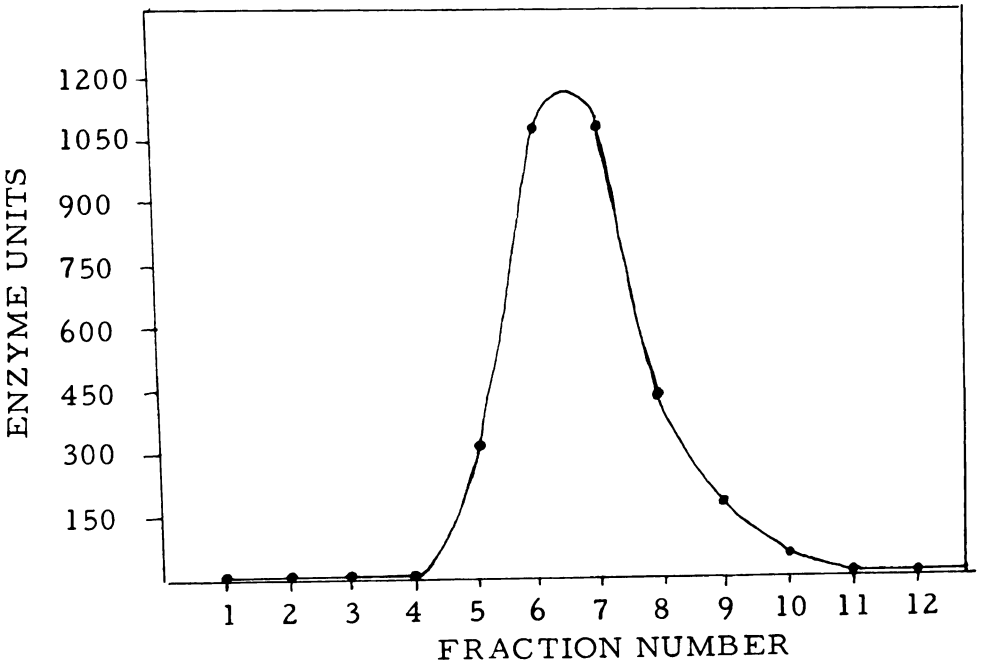
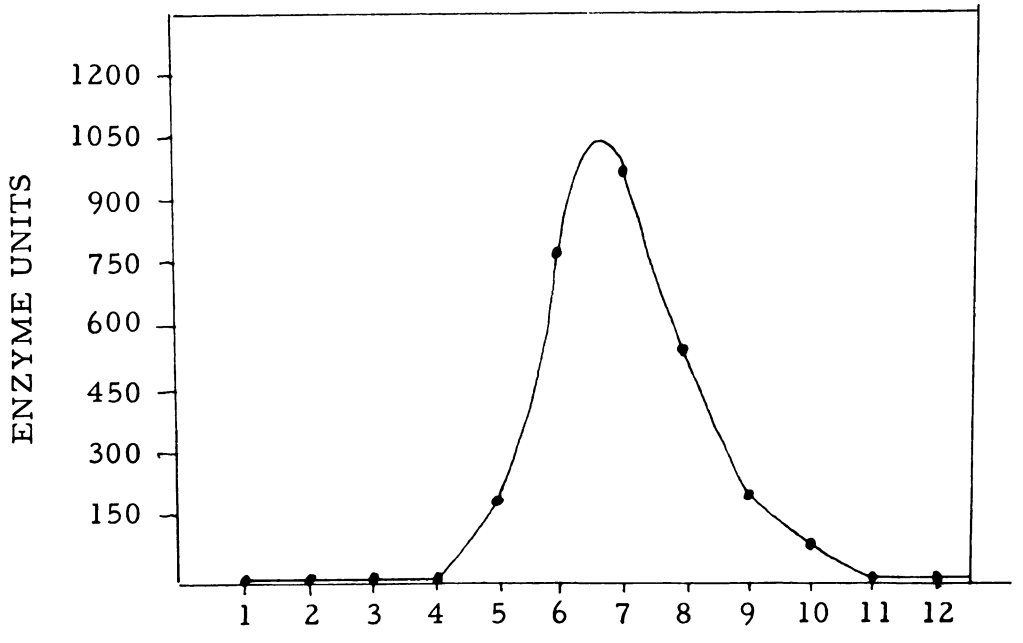


FIGURE 5B. Gel filtration column chromatography of dialyzed crude T^L extract in G-100 Sephadex.



Fine Chemicals, Inc.) in pH 8.6 barbital buffer and subjected to continuous-flow electrophoresis on two fresh sheets. Figure 6B demonstrates that the T^L3 isolated from the first electrophoretic separation remains most cathodic in its electrophoretic mobility while T^L2 remains slightly anodic.

C. ION EXCHANGE COLUMN CHROMATOGRAPHY

Column chromatography on DEAE cellulose of undialyzed T^L or T^S extracts in a sodium barbital buffer system (pH 8.6, I = 0.02; the same buffer used for continuous-flow electrophoresis) did not result in fractionation. All of the enzyme in both extracts was eluted completely in the break-through volume. However, resolution of the multiple forms of both T^L or T^S is revealed on DEAE cellulose in a sodium phosphate buffer system (see Materials and Methods). T^L and T^S are both fractionated into three components, but with different quantitative distributions. Figures 7A and 7B depict typical patterns. The elution patterns were the same whether the salt concentration was increased in a step-wise fashion or by means of a linear gradient. No additional enzyme was eluted in NaCl concentrations exceeding 0.5 M:

Enzyme eluted in the absence of NaCl will be called T^LA or T^SA; that eluted in 0.1M NaCl, T^LB or T^SB; and the 0.5M NaCl fraction, T^LC or T^SC. T^L extracts, as was the case with

FIGURE 6A. Analysis of fractions collected from bottom of continuous-flow electrophoresis sheet.

Sample: Crude undialyzed T^L extract

Sample Volume: 150 ml

Total Activity: 90,000 units

Activity Recovered: 32,160 (35.7%)

Time of Electrophoresis: 84 hours

FIGURE 6B. Analysis of fractions collected from two separate continuous-flow electrophoresis sheets.

Sample: $T^{L}3$; tubes (3-8) from Figure 4A

$T^{L}2$; tubes (10-15) from Figure 4A

Sample Volume: $T^{L}3$ = 32 ml

$T^{L}2$ = 20 ml

Total Activity: $T^{L}3$ = 7,680 units

$T^{L}2$ = 3,800 units

Activity Recovered: $T^{L}3$ = 2,445 (31.8%)

$T^{L}2$ = 681 (17.9%)

Time of Electrophoresis: $T^{L}3$ = 27 hours

$T^{L}2$ = 24 hours

FIGURE 6A. Continuous-flow electrophoresis analysis, T^L.

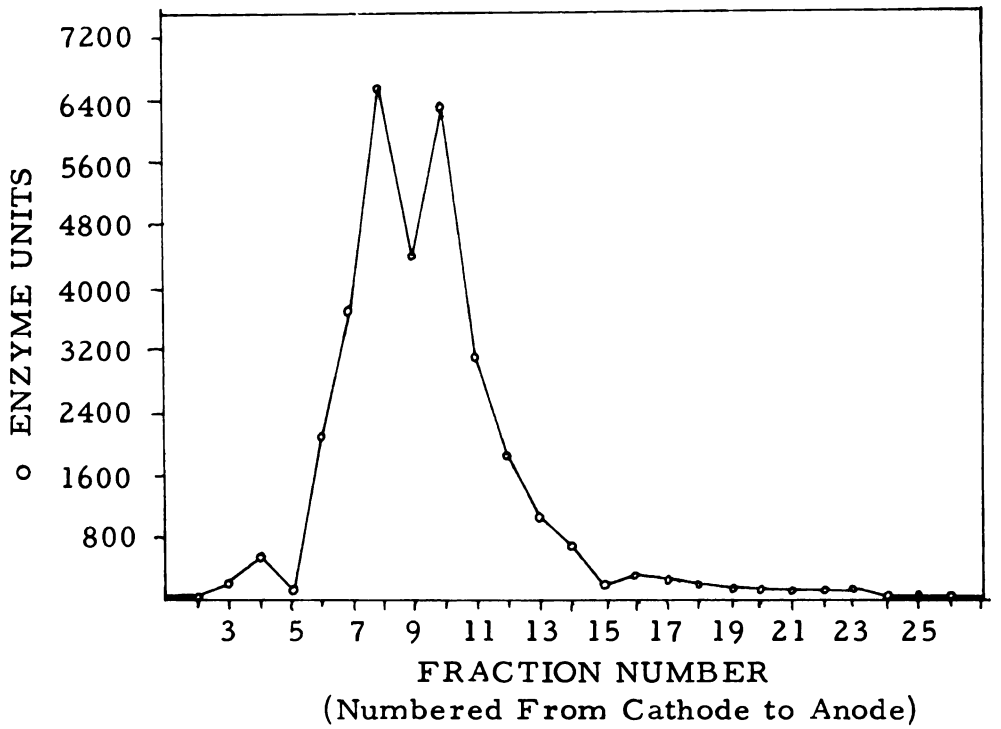
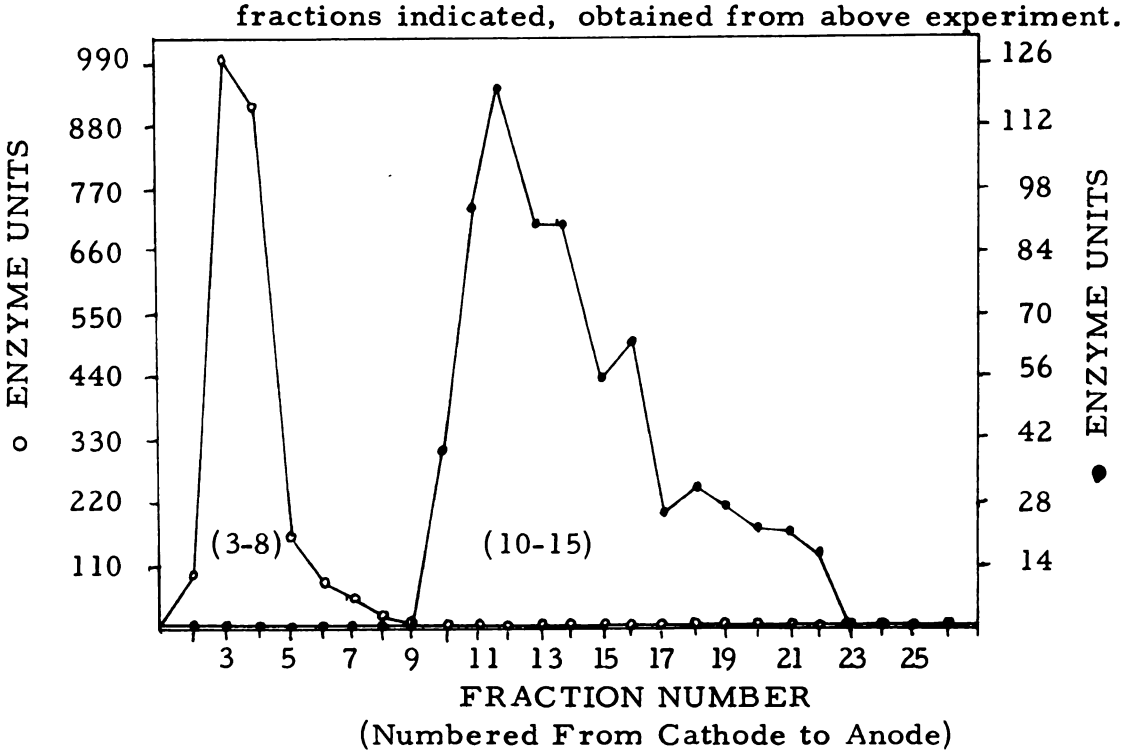


FIGURE 6B. Continuous-flow electrophoresis analysis of fractions indicated, obtained from above experiment.



continuous-flow electrophoresis, showed much more variability in the quantitative distribution of the three components than did the T^S extracts. However the ratio of $T^LB:T^LA$ was always greater than $T^SB:T^SA$. After buffer exchange by dialysis or gel filtration a shift to TA is evident in both extracts (compare Figures 7A and 8A, 7B and 8B, and Table 1).

It was observed that with either extract a greater percentage of TA was present when the extract was dialyzed in a bag overnight than when subjected to gel filtration. Since the only difference in principle between these two methods is time (20 minutes on Sephadex versus 24 hours in a bag) it was postulated that a spontaneous conversion to TA takes place. In order to test this possibility an extract was prepared, immediately passed through a G-25 Sephadex column, and an aliquot was chromatographed on DEAE cellulose. The rest of the extract was allowed to incubate in the cold and aliquots were chromatographed on DEAE cellulose after 24 and 48 hours. Table 2 illustrates the results of such an experiment using a T^S extract, and clearly shows that incubation of the filtered extract results in an increase of the T^SA component and losses of both T^SB and T^SC. These results are comparable to those obtained by Fox and Burnett (67), who showed a quantitative conversion of T1 and T2 to T3 by continuous-flow electrophoresis with their 131a-15300 strain of Neurospora crassa.

FIGURE 7A. Ion-exchange column chromatography of undialyzed crude T^L extract on DEAE cellulose.

Dimensions of Column Bed = 1.2 cm by 40.0 cm

Sample Size = 10 ml

Tyrosinase Activity Applied = 4,200 units

Tyrosinase Activity Recovered = 4,095 (97.1%)

Fraction Size = 9.0 ml

Method of Elution = Step-wise; 0.0M NaCl, 0.1M NaCl, and 0.5M NaCl

FIGURE 7B. Ion-exchange column chromatography of undialyzed crude T^S extract on DEAE cellulose.

Dimensions of Column Bed = 1.2 cm by 40.0 cm

Sample Size = 10 ml

Tyrosinase Activity Applied = 3,500 units

Tyrosinase Activity Recovered = 3,438 units (98.2%)

Fraction Size = 9.0 ml

Method of Elution = Step-wise; 0.0M NaCl, 0.1M NaCl, and 0.5M NaCl

FIGURE 7A. Ion-exchange column chromatography of undialyzed, crude T^L extract on DEAE cellulose.

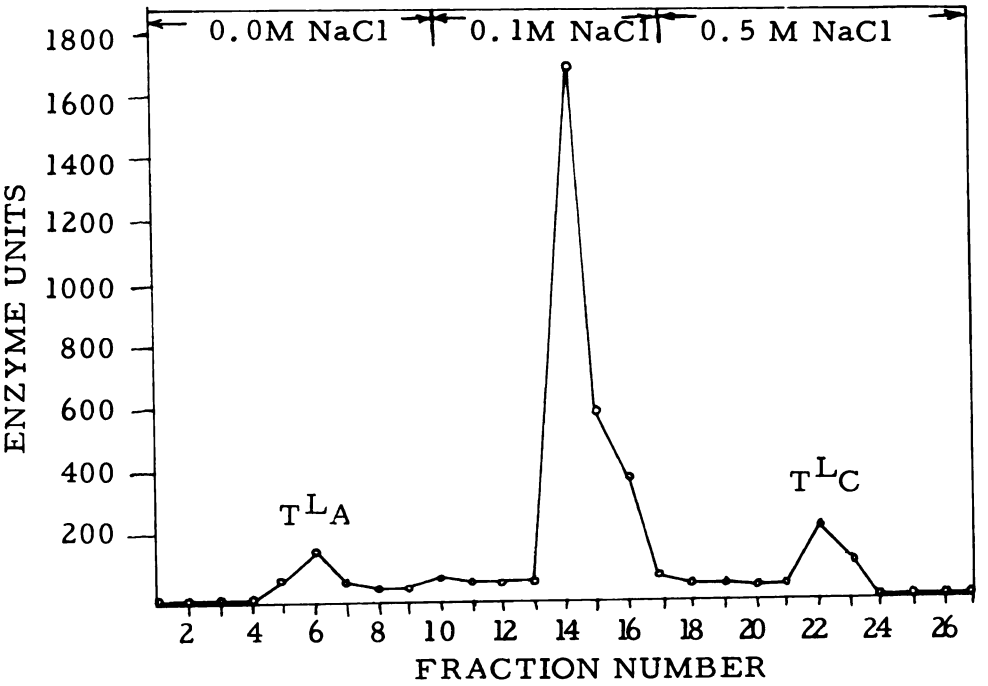


FIGURE 7B. Ion-exchange column chromatography of undialyzed, crude T^S extract on DEAE cellulose.

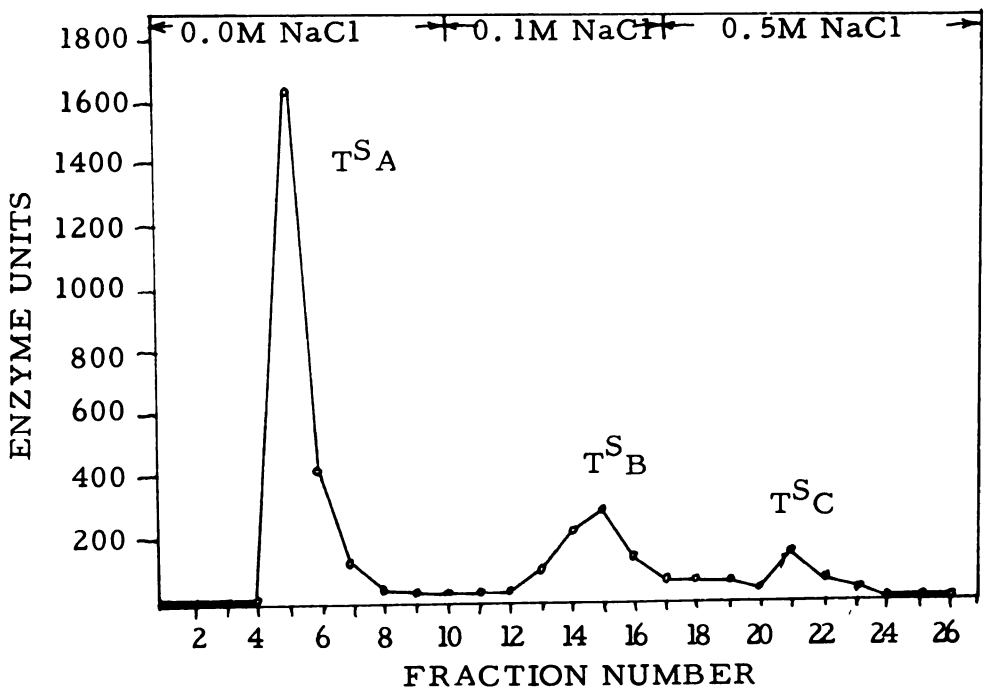


FIGURE 8A. Ion-exchange column chromatography of crude, T^L extract on DEAE cellulose after passage over G-25 Sephadex.

Dimensions of Column Bed = 1.2 cm by 40.0 cm

Sample Size = 10.0 ml

Tyrosinase Activity Applied = 4,200 units

Tyrosinase Activity Recovered = 3,636 units (86.6%)

Fraction Size = 9.0 ml

Method of Elution = Step-Wise; 0.0M NaCl, 0.1M NaCl, and 0.5M NaCl

FIGURE 8B. Ion-exchange column chromatography of crude, T^S extract on DEAE cellulose after passage over G-25 Sephadex.

Dimension of Column Bed = 1.2 cm by 40.0 cm

Sample Size = 7.5 ml

Tyrosinase Activity Applied = 2,600 units

Tyrosinase Activity Recovered = 2,520 units (96.9%)

Fraction Size = 9.0 ml

Method of Elution = Step-wise; 0.0M NaCl, 0.1M NaCl, and 0.5M NaCl

FIGURE 8A. Ion-exchange column chromatography of crude T^L extract on DEAE cellulose, after passage over G-25 Sephadex.

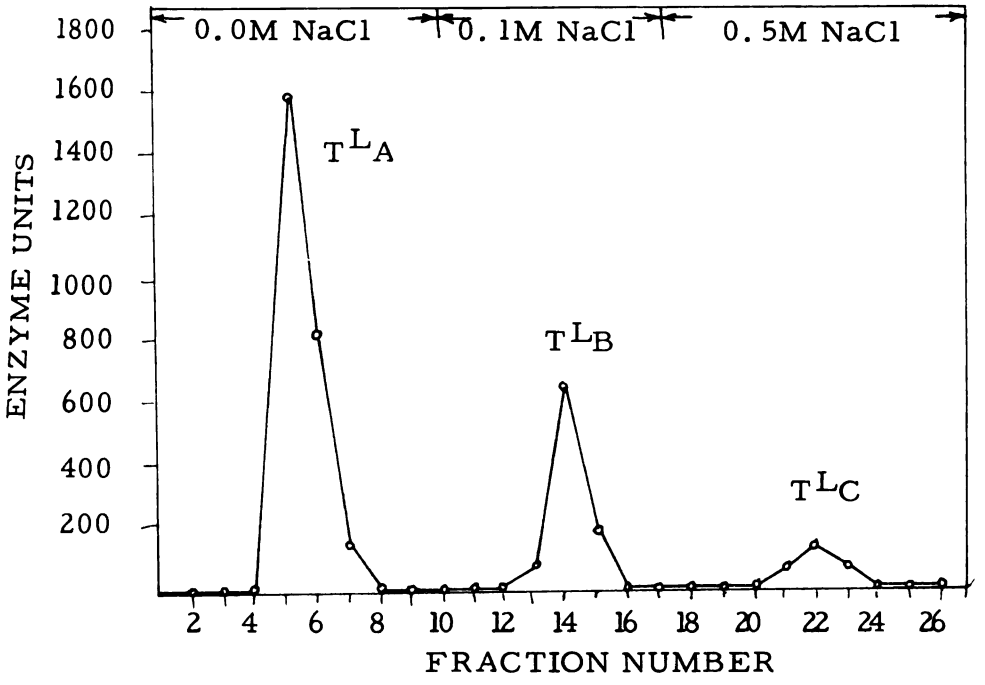


FIGURE 8B. Ion-exchange column chromatography of crude T^S extract on DEAE cellulose, after passage over G-25 Sephadex.

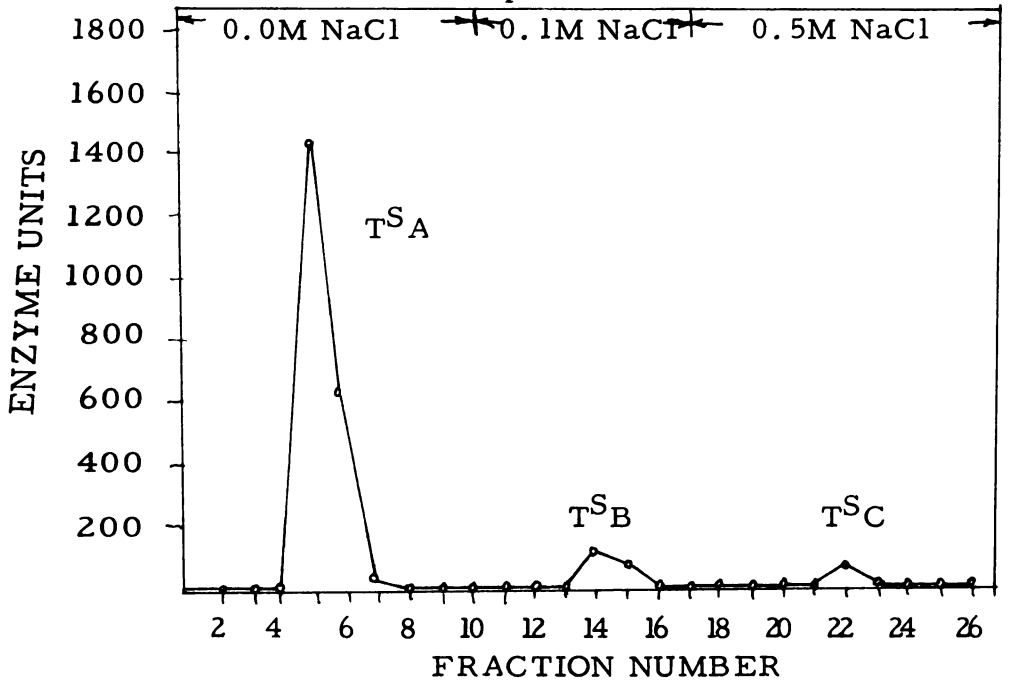


TABLE 1. Distribution of Multiple forms of tyrosinase before and after passage over G-25 Sephadex as determined by ion-exchange on DEAE cellulose for both T^L and T^S.

	T^L_A	T^L_B	T^L_C
Before gel filtration (percent ot total)	14.7	75.8	9.5
After gel filtration (percent of total)	73.0	23.5	3.5

	T^S_A	T^S_B	T^S_C
Before gel filtration (percent of total)	68.8	23.3	7.9
After gel filtration	85.1	11.4	3.5

TABLE 2. Changes in tyrosinase during incubation of T^S extract after passage over G-25 Sephadex as determined by ion-exchange chromatography on DEAE cellulose.

Length of Incubation at 5° C. (hours)	T^S_A		T^S_B		T^S_C		Total
	Units	Percent	Units	Percent	Units	Percent	Units
0	1314	81.6	207	12.8	90	5.6	1611
24	1467	93.5	51	3.2	51	3.2	1569
48	1719	95.0	45	2.5	45	2.5	1809

Six molar urea did not change the DEAE cellulose elution patterns of either T^L or T^S extracts (Figure 9 and Table 3), nor was any change observable in the presence of various concentrations of cupric nitrate, cuprous nitrate, or ethylenedinitrilotetraacetic acid (EDTA).

Crude Neurospora extracts usually contained between 4-5 mg of protein per ml. This protein was eluted in a ratio of 30:50:20 in the three fractions eluted from the DEAE cellulose columns used. Recovery of total protein from these columns was between 70 and 80 percent. Total tyrosinase recovery was generally 90-100 percent. TA, TB, and TC tyrosinase components were purified approximately 18-fold, 12-fold, and 16-fold respectively. A second passage over DEAE cellulose increased the purity to approximately 25 fold.

Rechromatography of either $T^L B$ and $T^S B$ on DEAE cellulose after dialysis or gel filtration to remove the NaCl always resulted in conversion, so that at least 98 percent of the original TB component was recovered as TA. In addition, direct cross identification between the enzyme components obtained electrophoretically and those obtained chromatographically proved impossible because of the necessity to change buffers by dialysis or gel filtration. Thus when $T^L 3$, $T^L 2$, and $T^L 1$ (fractions 5-9, 10, and 11-22, respectively, from Figure 2) were individually chromatographed on DEAE cellulose after dialysis or gel filtration against sodium phosphate buffer

FIGURE 9. Ion-exchange column chromatography on DEAE cellulose of T^L extract treated with 6M urea.

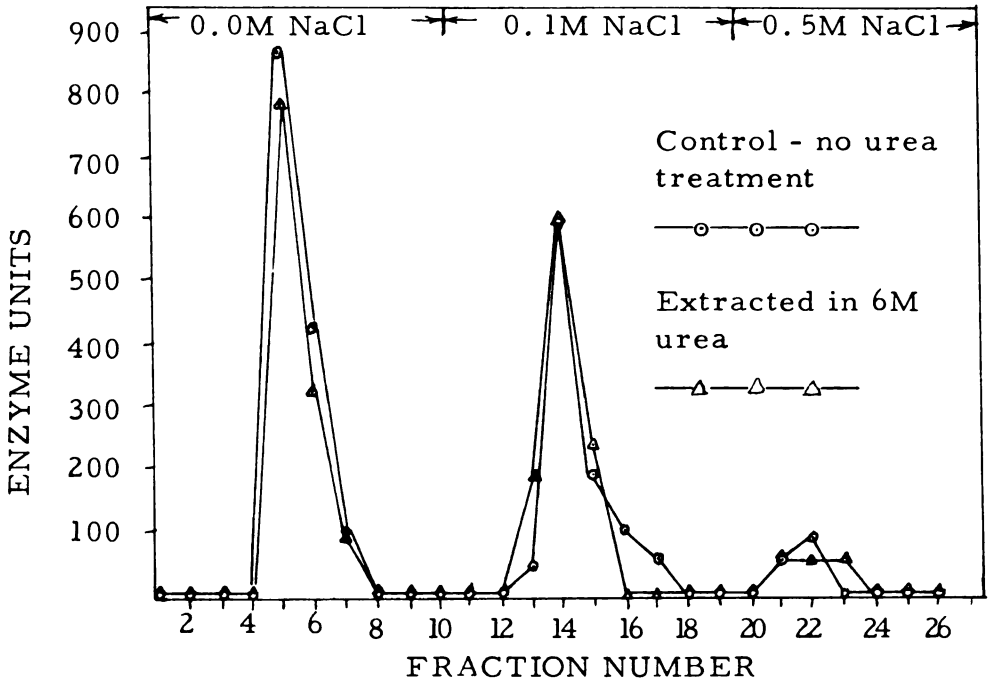


TABLE 3. Affect of extraction in 6M urea on the distribution of tyrosinases (T^L) as determined by ion-exchange column chromatography on DEAE cellulose.

Fraction	Control Percent of Total	Urea Extracted Percent of Total
T^L_A	58.7	50.0
T^L_B	37.0	44.0
T^L_C	4.3	6.0

(pH 8, 0.11M), they were all recovered predominately as $T^{L}A$ (Figure 10). Similarly, $T^{L}B$ exhibited the electrophoretic properties of $T^{L}3$ after dialysis or gel filtration against sodium barbital buffer (pH 8.6, I = 0.02). There is sufficient indirect evidence, however, to say with some assurance that TA is equivalent to T3, that TB is equivalent to T2, and the TC is equivalent to T3, and that these difficulties are attributable to conversion (see Discussion).

D. THERMAL INACTIVATION

Thermal inactivation of $T^{L}A$ and $T^{S}A$ has been examined. In all cases inactivation exhibited first order kinetics. Inactivation curves observed at three temperatures are illustrated in Figure 11. Tables 4 and 6 give the thermal half-lives of $T^{L}A$ and $T^{S}A$ calculated by means of the least-square method from data such as is shown in Figure 11. The rate constants of inactivation are given in Tables 5 and 7. A typical Arrhenius plot of these data is shown in Figure 12.

The conversion of $T^{L}B$ to $T^{L}A$ upon dialysis or gel filtration prevented an unambiguous determination of its thermal stability. However, it was possible to compare authentic $T^{L}A$ with $T^{L}A$ obtained by the conversion of $T^{L}B$ (referred to as $T^{L}B \rightarrow T^{L}A$). To accomplish this comparison, crude T^{L} extract was chromatographed on DEAE cellulose and $T^{L}A$ and $T^{L}B$ were isolated. $T^{L}A$

FIGURE 10. Ion-exchange column chromatography on DEAE cellulose of continuous flow electrophoresis fractions from T^L. See Figure 2.

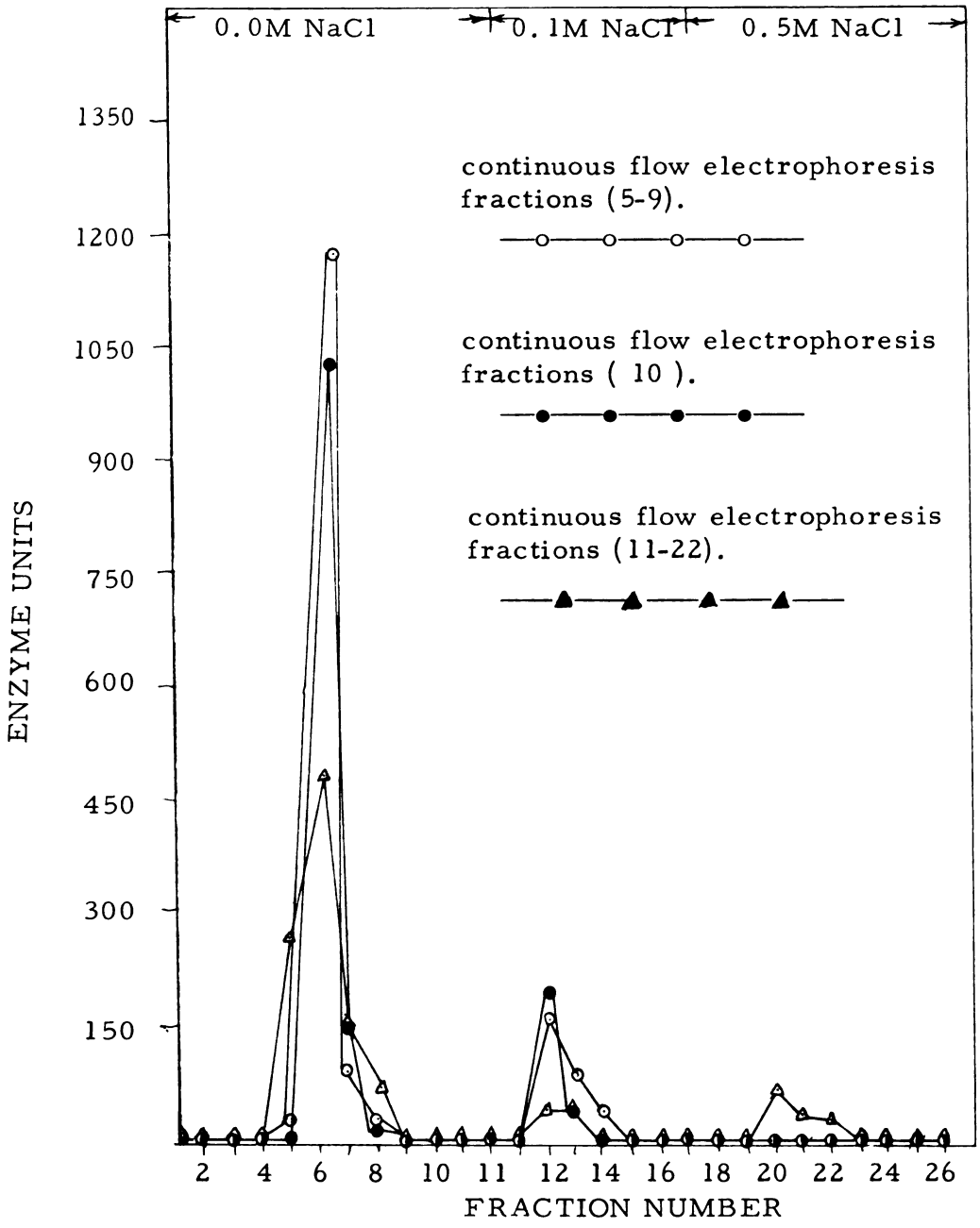


TABLE 4. Thermal half-lives of $T^{L}A$ and $T^{L}B \rightarrow T^{L}A$ in minutes. Determined in 0.1M sodium phosphate buffer, pH 6.0.

	Temperature		
Tyrosinase	50°	55°	59°
$T^{L}A$	215.0	42.4	10.4
$T^{L}B \rightarrow T^{L}A$	200.1	30.1	9.5

TABLE 5. Rate constants of thermal inactivation (k) of $T^{L}A$ and $T^{L}B \rightarrow T^{L}A$ from half-lives shown in Table 4.
$\mu_{inact.}$ = activation energy of thermal inactivation in calories per mole.

	$k \times 10^{2}$ minute^{-1}			
	Temperature			
Tyrosinase	50°	55°	59°	$\mu_{inact.}$
$T^{L}A$	0.32	1.63	6.68	76,440 ± 3,621
$T^{L}B \rightarrow T^{L}A$	0.35	2.26	7.30	75,799 ± 9,911

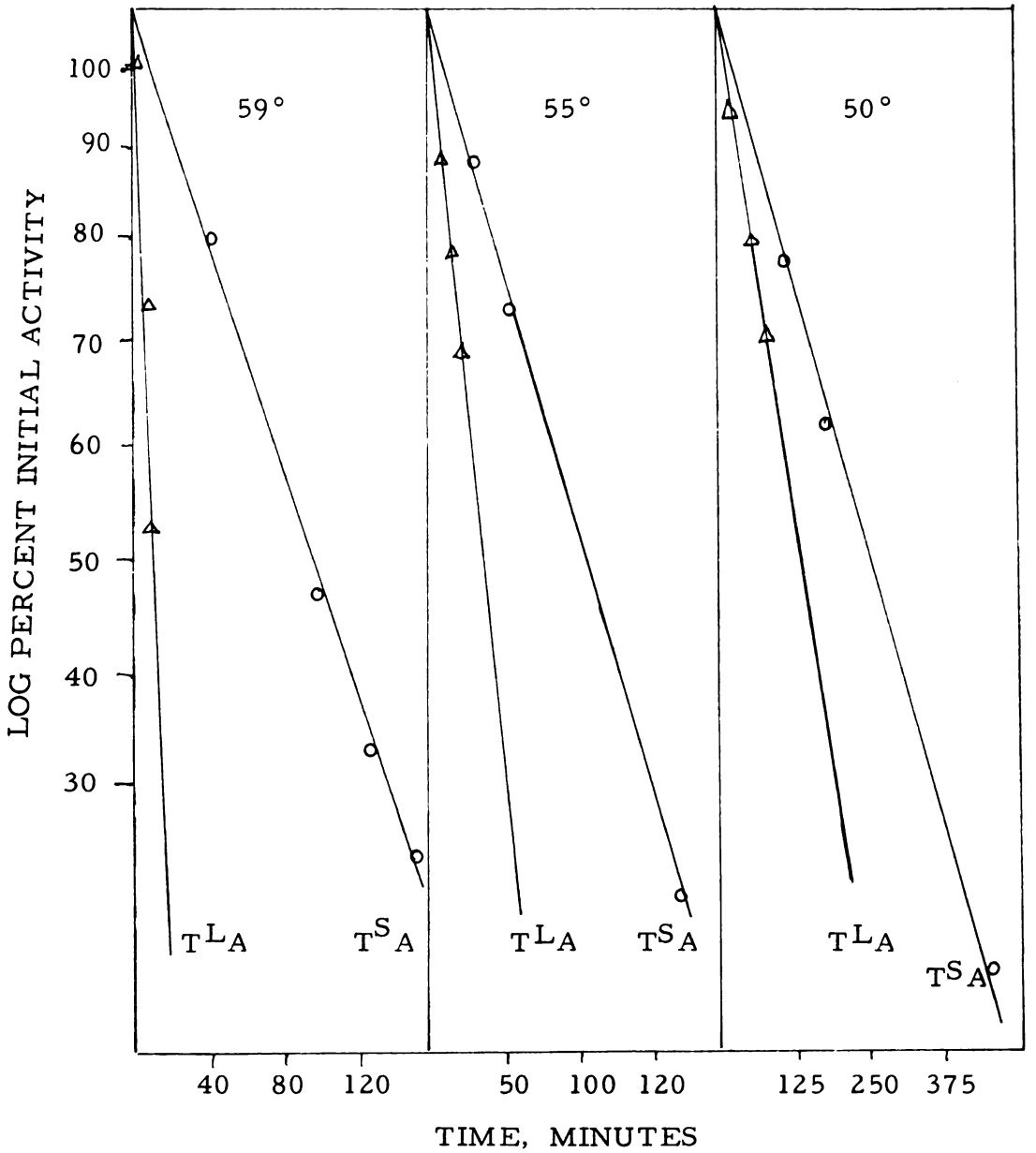
TABLE 6. Thermal half-lives of tyrosinase $T^S A$ in sodium phosphate buffer, pH 6.0 and $T^S 3$ in barbital buffer, pH 8.6, in minutes.

	Temperature		
Tyrosinase	48°	55°	59°
$T^S A$	301.00	100.30	75.25
$T^S 3$	2.18	1.31	0.85

TABLE 7. Rate constants of thermal inactivation (k) of $T^S A$ and $T^S 3$ from half-lives shown in Table 6. $\mu_{inact.}$ = activation energy of thermal inactivation in calories per mole.

	$k \times 10^3$ minute^{-1}			
	Temperature			
Tyrosinase	48°	55°	59°	$\mu_{inact.}$
$T^S A$	2.3	6.9	9.2	27,113
$T^S 3$	318.0	529.0	812.0	21,755

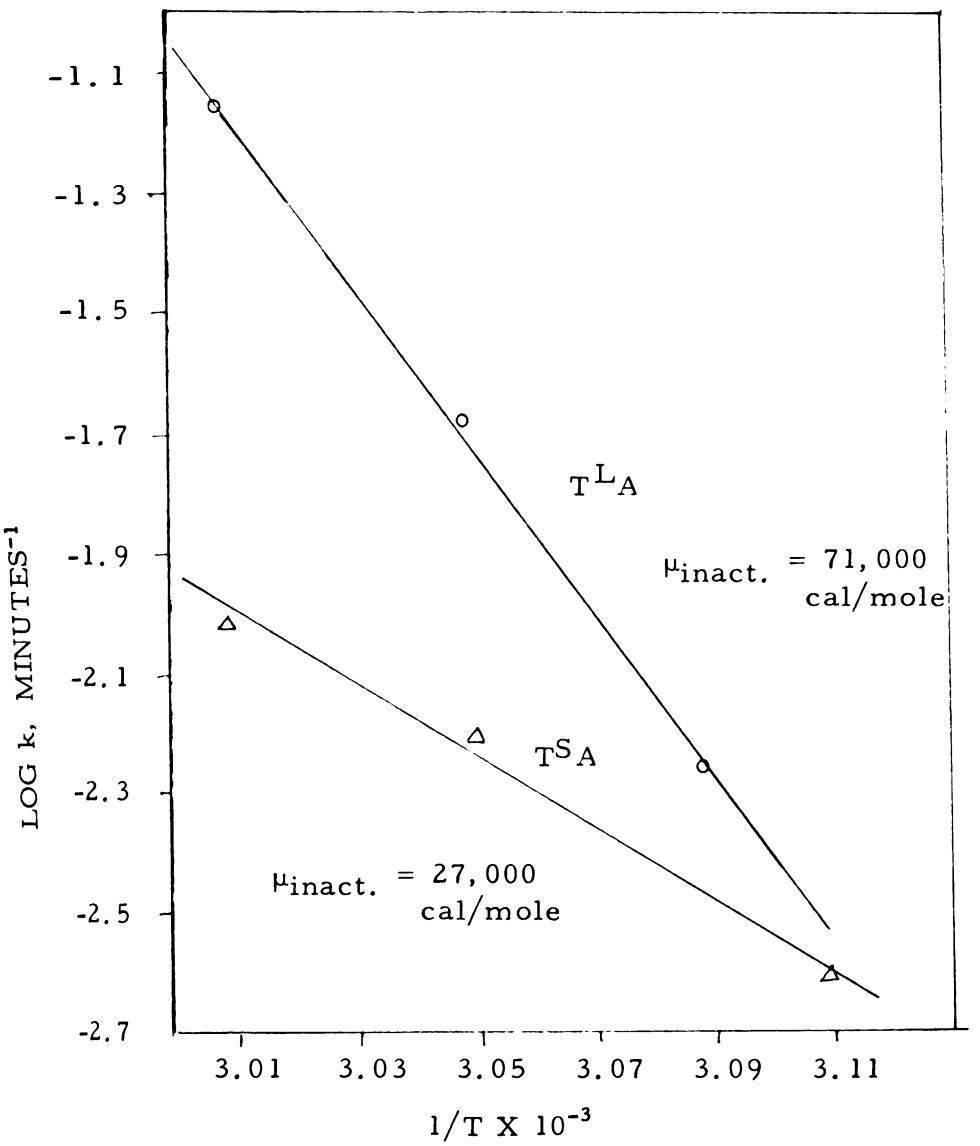
FIGURE 11. Thermal inactivation of $T^{L}A$ and $T^{S}A$ at three temperatures. Ordinate: residual activity plotted on a logarithmic scale. Abscissae: time of exposure to the indicated temperature.



was passed through a G-25 Sephadex column previously equilibrated with 0.1M sodium phosphate buffer, pH 6.0. $T^{L}B$ was passed over G-25 Sephadex, but in a column equilibrated with 0.1M sodium phosphate buffer pH 8.1. The enzyme was then rechromatographed on a new DEAE cellulose and was recovered as $T^{L}A$; the $T^{L}B \rightarrow T^{L}A$ conversion had occurred. This was passed through a G-25 Sephadex column equilibrated with 0.1M sodium phosphate buffer, pH 6.0, in order to provide ionic conditions similar to those of authentic $T^{L}A$. Thermal half-lives of the two preparations were determined at three different temperatures and their respective activation energies of inactivation were calculated (Tables 4 and 5). There was no significant difference, and the values agree reasonably well with those reported for T^{L} by Sueoka (59).

The thermal half-lives at three temperatures of $T^{S}A$ in 0.1M sodium phosphate buffer, pH 6.0, were determined and are given in Table 6. The half-life of $T^{S}A$ at 59°C. (75 minutes) is consistent with Sueoka's value (65 minutes) for both crude and partially purified T^{S} (59). $T^{S}3$ obtained from a continuous-flow electrophoresis experiment was also tested for thermal inactivation. The determination was carried out in pH 8.6 barbital buffer (Table 6). In spite of the differences in their thermal half-lives at the temperatures tested, the activation energy of inactivation for $T^{S}A$ and $T^{S}3$ were the same (Table 7). This value, however, is very

FIGURE 12. Arrhenius plot of the rate constants of thermal inactivation of $T^{L}A$ and $T^{S}A$. k = rate constant; T = absolute temperature. μ_{inact} = activation energy of inactivation in calories per mole.



different from the one reported by Sueoka (59). A fuller discussion of this point will be found in the next section.

The thermal inactivation data clearly illustrate that $T^{L}A$ and $T^{S}A$ are different (Figure 12). Figure 13 also clearly shows that $T^{L}B$ and $T^{S}B$ are different. Thermal inactivation at 59° C. of $T^{L}B$ and $T^{S}B$ was studied immediately after collection from separate DEAE cellulose columns. The fractions were not dialyzed or treated in any way, so that both determinations were carried out in the same buffer with the same NaCl concentration. $T^{S}A$ and $T^{S}B$, respectively, are more heat stable than $T^{L}A$ and $T^{L}B$.

E. SUCROSE GRADIENT CENTRIFUGATION

Sucrose gradient centrifugation of undialyzed and dialyzed T^{S} extracts at pH 8.0 in 0.1M sodium phosphate buffer, shows a single symmetrical peak of tyrosinase activity for each, with the same S value (Figure 14). From the position of the bovine hemoglobin standard the molecular weight is estimated to be about 67,500, which agrees with Horowitz's determination (58).

Figure 15 compares undialyzed T^{L} and T^{S} extracts at pH 8.0 in 0.1M sodium phosphate buffer and again reveals a single peak of activity for each in the same place.

The sucrose gradient centrifugation data was confirmed by gel filtration with G-100 and G-200 Sephadex.

FIGURE 13. Thermal inactivation of $T^{L}B$ and $T^{S}B$ at 59° C. Ordinate: residual activity plotted on a logarithmic scale. Abscissae; time of exposure to indicated temperature. t 1/2 = half-life.

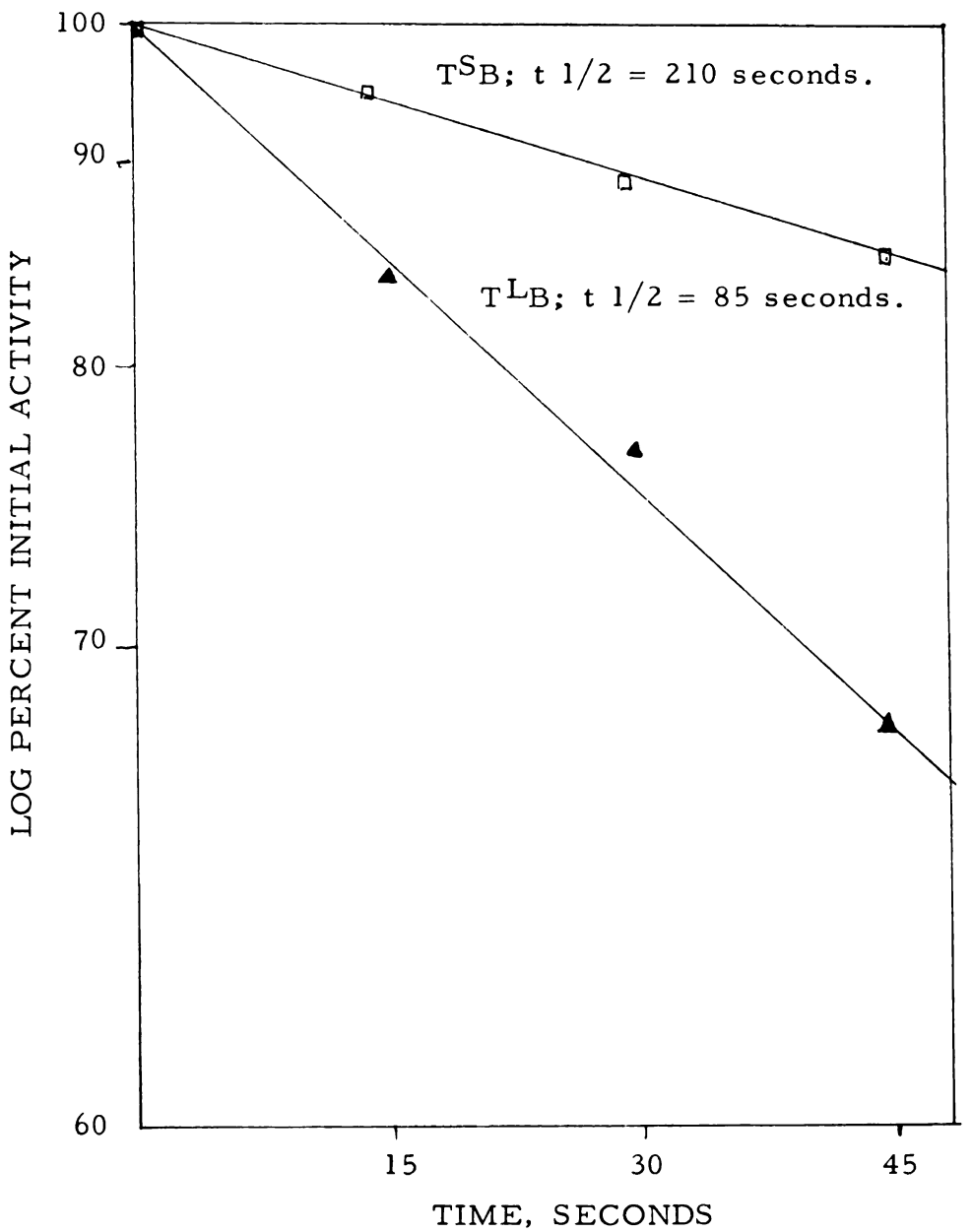


FIGURE 14. Sedimentation patterns of crude, dialyzed and undialyzed T^S extract and crystallized bovine hemoglobin in a linear 5 to 20 percent sucrose gradient.

Sample Sizes: Dialyzed T^S = 0.2 ml

Undialyzed T^S = 0.2 ml

Hemoglobin Standard = 0.2 ml (2.0 mg)

Centrifuged for 24 hours in a Spinco SW 39 rotor at 34,000 rpm at 5 C, in sodium phosphate buffer, 0.1M, pH 8.0.

Total volume for all tubes was 5.3 ml

FIGURE 14. Sedimentation patterns of crude dialyzed and undialyzed T^S extract and crystallized bovine hemoglobin in a linear 5 to 20 percent sucrose gradient.

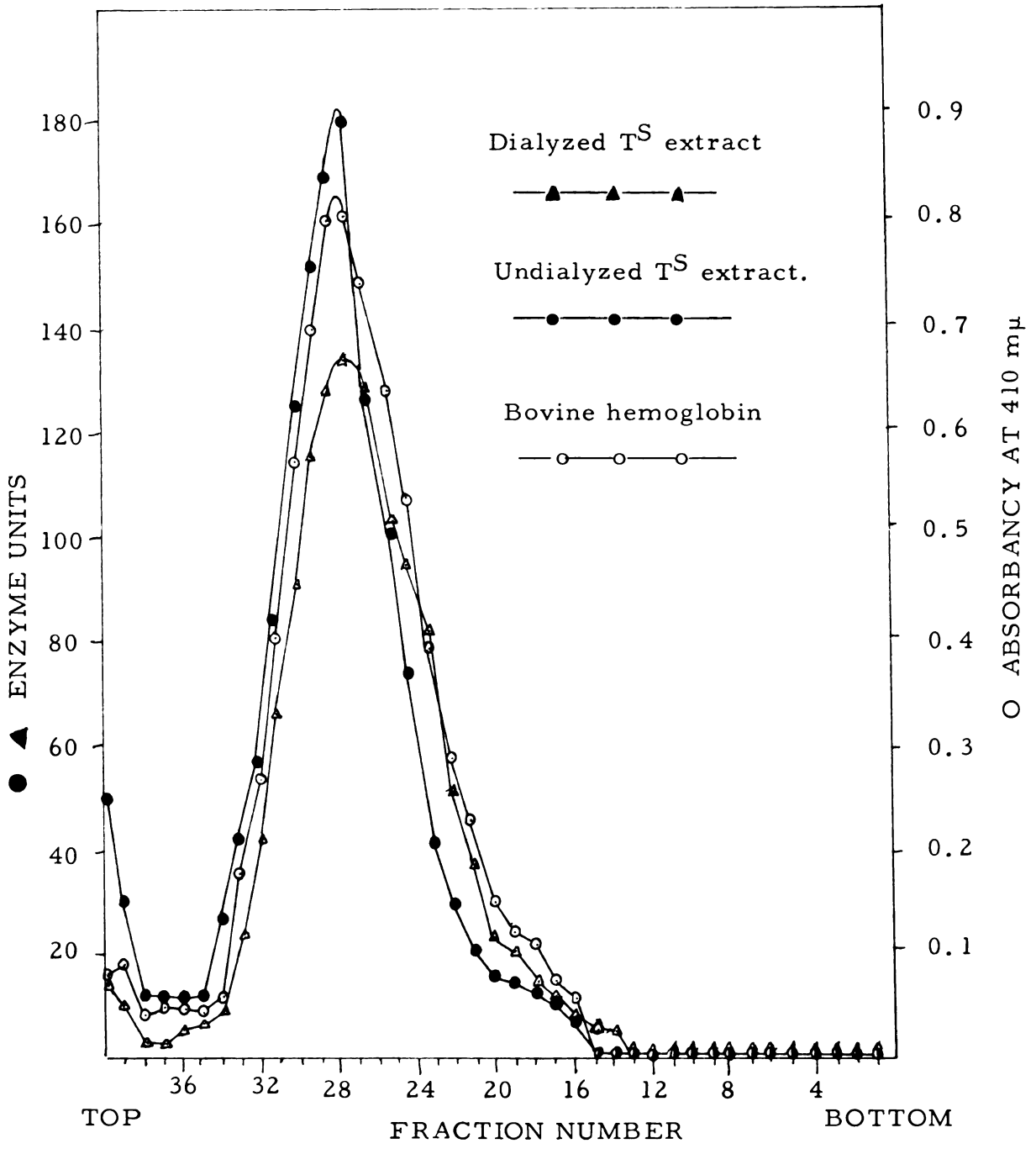


FIGURE 15. Sedimentation patterns of crude T^L and T^S extract in a linear 5 to 20 percent sucrose gradient.

Sample Sizes: T^L = 0.2 ml

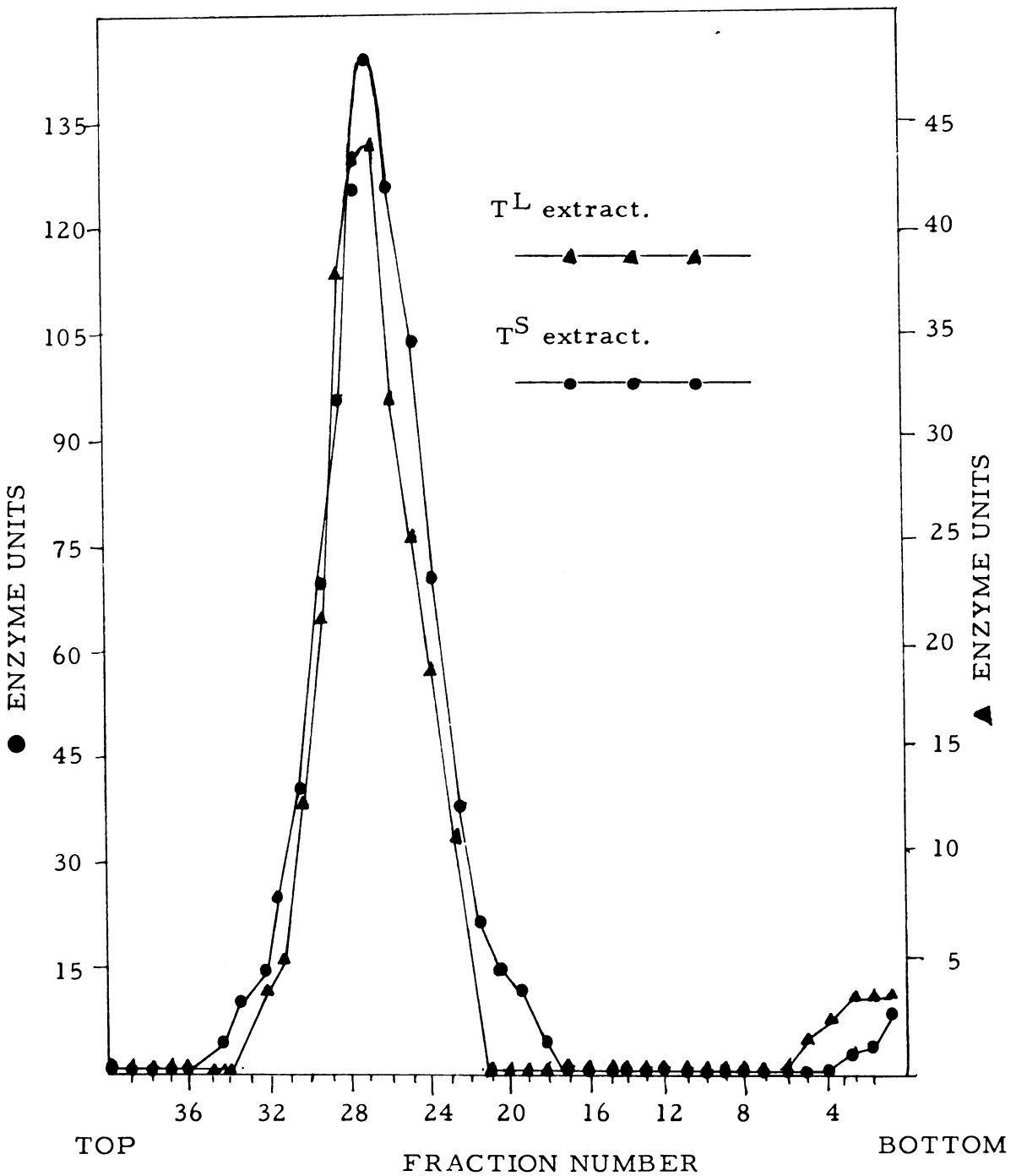
T^S = 0.2 ml

Hemoglobin Standard (Not shown) = 0.2 ml (2.0 mg)

Centrifuged for 24 hours in a Spinco SW 39 rotor at 34,000 rpm at 5° C, in sodium phosphate buffer, 0.1M, pH 8.0.

Total volume for all tubes was 5.3 ml

FIGURE 15. Sedimentation patterns of crude T^L and T^S extract in a linear 5 to 20 percent sucrose gradient.



DISCUSSION AND CONCLUSIONS

The evidence presented in this study clearly indicate the presence of multiple forms of tyrosinase in strains T^S and T^L, and their convertibility. The phenomena originally reported by Fox and Burnett (67) in a different strain of Neuospora crassa have been confirmed. Interconversion of different tyrosinases is not restricted to Neurospora. Jolley and Mason (82) reported a similar phenomenon among the multiple forms of mushroom tyrosinases. Unlike horse heart cytochrome c (83), the conversion of Neurospora tyrosinase is not due to protein aggregation and dissociation.

Study of the multiple forms of tyrosinase in Neurospora was made extremely difficult by their instability. The most pronounced difference between T^S and T^L in their quantitative distribution was observed in crude extracts. Gel filtration and dialysis promoted conversion in both extracts to the same electrophoretic and chromatographic form even though this component differed with regard to heat stability. Dialysis also relieved an apparent inhibition of tyrosinase found in all extracts. Indeed, it may be suggested that the multiple forms themselves are artifactual and due to differential association with a small molecule or ion acting as an inhibitor. In fact, however, T^L3 and T^L2 do not exhibit

apparent inhibition and when they were subjected to re-electrophoresis under conditions which did not change their environment after the original fractionation (i.e. after dialysis against Ficoll), they remained stable and did not change their electrophoretic positions. The inhibitor therefore must be ruled out as the cause of the multiple forms. In addition, conversion of $T^{L}B$ to $T^{L}A$ and $T^{S}B$ to $T^{S}A$ proceeded even though the inhibitor effect was removed by previous dialysis or gel filtration. The considerations presented here do not rule out the possibility of the association of tyrosinase with another small molecule or even another protein. The important point, however, is that the multiple forms exist regardless of the uncertainty about the basis of their differences. The significance of this statement is strengthened by the fact that the distributions of the multiple forms is different in T^{L} and T^{S}, two strains with a known genetic difference. It is probable that the three tyrosinases produced by each strain differ in tertiary configuration.

The data strongly suggest that T_3= TA, T2 = TB, and T1 = TC, even though a direct demonstration was not possible. The proportions and order of appearance of TA, TB, and TC upon elution from DEAE cellulose are what one would predict from the distribution of T1, T2, and T3 after continuous-flow electrophoresis. Electrophoresis reveals $T^{L}2 > T^{L}3 > T^{L}1$ and $T^{S}3 > T^{S}2 > T^{S}1$, while DEAE cellulose chromatography shows $T^{L}B > T^{L}A > T^{L}C$ and $T^{S}A > T^{S}B > T^{S}C$. With

regard to charge, T1 is more negative than T2 and T2 is more negative than T3, while TC is more negative than TB and TB is more negative than TC. T2 and T1 are converted to T3, while TB and TC are converted to TA. Moreover the 131a-15300 strain of Neurospora crassa produces a tyrosinase indistinguishable from the T^S tyrosinase. Fox and Burnett (67) reported that the proportions of T1, T2, and T3 in this strain were 5.9, 12.8, and 81.3%, respectively, of the total tyrosinase content in fresh extracts as determined by continuous-flow electrophoresis. They also showed that the conversion, after incubation, of T1 and T2 to T3 was quantitative. In fresh extracts TC, TB, and TA constitute 5.6, 12.8, and 81.6% of the total tyrosinase, respectively. The conversion of TB and TC to TA, after incubation was shown in this study also to be quantitative.

The thermal inactivation data of tyrosinase obtained with the T^L strain agrees reasonably well with that reported by Horowitz's group. A major discrepancy exists with regard to the T^S strain. The activation energy of inactivation for T^S has been given by Horowitz and Fling (56) and Sueoka (59) as 60,000 calories per mole. The value obtained in this study was 27,000 calories per mole. Using the thermal half-lives for T^S at three different temperatures given by Horowitz in an early paper (55), the activation energy of inactivation was calculated to be 25,000 calories per mole, a

figure in agreement with that reported here. The reason for these discrepancies is unknown.

Based on the data obtained herein, Fox's suggestion that the T locus controls the equilibrium in the interconversion of the alternate forms of tyrosinase (67, 68) must be abandoned. It can also be concluded that the multiple forms found in both T^L and T^S are not due to the presence of different size protein aggregates. In addition differences in molecular weights do not form the basis of an explanation for the differences in heat stability between T^L and T^S. The reason for the heat stability difference is still unknown. A difference in tertiary structure between the L form of tyrosinase and the S form is not excluded by anything reported here or elsewhere. Horowitz's suggestion that the difference is attributable to differences in primary structure must await a complete sequence analysis for verification.

SUMMARY

1. Multiple forms of tyrosinase were found in extracts of two strains of Neurospora crassa (T^S and T^L).

2. Three forms of tyrosinase were observed in each of the strains by continuous-flow electrophoresis. The quantitative distribution of the three forms was different in the two strains. The forms were designated T1, T2, and T3.

3. In both strains, conversion of T1 and T2 to T3 occurred after dialysis or gel filtration.

4. Ion-exchange column chromatography also reveals three forms in both strains. These were designated TA, TB, and TC, and the quantitative distribution paralleled that found by continuous-flow electrophoresis.

5. Dialysis or gel filtration of the extracts before chromatography results in a conversion of both TB and TC to TA in both strains.

6. In T^S extract the conversion is quantitative.

7. Direct attempts to cross identify the electrophoretic and chromatographic forms were frustrated, but a variety of indirect

evidences indicates that TA=T3, TB=T2, and TC=T1.

8. Gel-filtration with G-100 and G-200 Sephadex and sucrose gradient centrifugation of dialyzed and undialyzed T^L and T^S extracts show only one molecular species with a molecular weight of approximately 67,000-68,000. This result was interpreted to mean that the multiple forms of tyrosinase found in T^L and T^S are not a result of differential protein aggregation. In addition, the difference in heat stability between T^L and T^S cannot be explained by differences in molecular weight.

9. Thermal inactivation studies of some of the multiple forms of T^L and T^S tyrosinases indicate that the multiple forms themselves do not account for the heat stability differences between T^L and T^S.

REFERENCES

1. Crick, F.H.C., 1958. On Protein Synthesis. Symp. Soc. Exper. Biol., 12: 138-163.

2. Spiegelman, S., 1960. Factors Modulating the Biochemical Expression of Genetic Systems, in GENETICS, TRANSACTION OF THE FIRST CONFERENCE ed. by H. Eldon Sutton, p. 177, Josiah Macy, Jr. Foundation, New York.

3. Jacob, F., and Monod, J., 1961. On the Regulation of Gene Activity. Cold Spring Harbor Symp. Quant. Biol., 26: 193-211.

4. Levinthal, C., 1960. Genetic Control of Protein Structure, in GENETICS, TRANSACTION OF THE FIRST CONFERENCE ed. by H. Eldon Sutton, pp. 106-107, Josiah Macy, Jr. Foundation, New York.

5. Epstein, C.J., Goldberger, R.F., and Anfinsen, C.B., 1963. The Genetic Control of Tertiary Protein Structure: Studies with Model Systems. Cold Spring Harbor Symp. Quant. Biol., 28: 439-449.

6. White, F.H., Jr., 1961. Regeneration of Native Secondary and Tertiary Structures by Air Oxidation of Reduced Ribonuclease. J. Biol. Chem., 236: 1353-1360.

7. Epstein, C.J., Goldberger, R.F., Young, D.M., and

Anfinsen, C.B., 1962. A Study of the Factors Influencing the Rate and Extent of Enzymic Reactivation During Reoxidation of Reduced Ribonuclease. Arch. Biochem. Biophys., Suppl. 1: 223-231.

8. Goldberger, R.F., and Epstein, C.J., 1963. Characterization of the Active Product Obtained by Oxidation of Reduced Lysozme. J. Biol. Chem., 238: 2988-2991.

9. Imai, K.T., Takagi, T., and Isemura, T., 1963. Recovery of the Intact Structure of Muramidase (Lysozyme) After Reduction of all Disulfide Linkages in 8M Urea. J. Biochem., 53: 1-6.

10. Isemura, T. Takagi, T., Maeda, V., and Yutani, K., 1963. Recovery of the Intact Structure of Taka-Amylase A After Reduction of all Disulfide Bonds in 8M Urea. J. Biochem., 53:155-161.

11. Epstein, C.J., and Anfinsen, C.B., 1962. The Reversible Reduction of Disulfide Bonds in Trypsin and Ribonuclease Coupled to Carboxymethyl Cellulose. J. Biol. Chem., 237: 2175-2179.

12. Epstein, C.J., and Anfinsen, C.B., 1962. Reversible Reduction and Reoxidation of Poly-DL-Alanyl Trypsin. J. Biol. Chem., 237: 3464-3467.

13. Levinthal, C.E., Signer, R., and Fetheholt, K., 1962.

Reactivation and Hybridization of Reduced Alkaline Phosphatase. Proc. Natl. Acad. Sci., 48: 1230-1237.

14. Karusch, F., 1961. Disulfide Pairing and the Biosynthesis of Antibody, in IMMUNOCHEMICAL APPROACHES TO PROBLEMS IN MICROBIOLOGY ed. by M. Heidelberger and O.J. Plescia, pp. 368-376, Rutgers University Press, New Brunswick.

15. Steiner, R.F., DeLorenzo, F., and Anfinsen, C.B., 1965. Enzymically Catalyzed Disulfide Interchange in Randomly Cross-Linked Soybean Trypsin Inhibitor. J. Biol. Chem., 240: 4648-4651.

16. Isemura, T. and Imanishi, A., 1962. Reversibility of Denaturation and Molecular Stability of Bacillus subtilis α-amylase. J. Biochem., 50: 172-180.

17. Deal, W.C., Rutter, W.J., Massey, V., and Van Holde, K.E., 1963. Reversible Alterations of the Structure of Enzymes in Acidic Solution. Biochem. Biophys. Res. Commun., 10: 49-54.

18. Merigan, T.C. and Dreyer, W.J., 1963. Studies on the Antigenic Combining Sites in Bacteriophage Lysozyme. Ann. N.Y. Acad. Sci., 103: 765-772.

19. Merigan, T.C., and Dreyer, W.J., 1965. Serological Studies of Conformational Changes in a highly Helical Enzyme.

Biology Annual Report, Calif. Inst. of Technology, pp. 139-140.

20. Sela, M., and Lifson, S., 1959. On the Reformation of Disulfide Bridges in Proteins. Biochem. Biophys. Acta, 36: 471-478.

21. Helinski, D.R., and Yanofsky, C., 1966. Genetic Control of Protein Structure, in THE PROTEINS ed. by H. Neurath, 2nd ed., pp. 1-93, Academic Press, New York.

22. Berson, S.A., and Yalow, R.S., 1961. Immunochemical Distinction Between Insulins with Identical Amino-Acid Sequences. Nature, 191: 1392-1393.

23. Berson, S.A., and Yalow, R.S., 1963. Antigens in Insulin Determinants of Specificity of Porcine Insulin in Man. Science, 139: 844-845.

24. Perutz, M.F., Rossamann, M.G., Cullis, A.F., and Muirhead, H., 1960. Structure of Haemoglobin. Nature, 185: 416-422.

25. Kendrew, J.C., Dickerson, R.E., Strandberg, B.E., Hart, R.G., and Davic, D.R., 1960. Structure of Myoglobin. Nature, 185: 422-427.

26. Watson, H.C., and Kendrew, J.C., 1961. Comparison Between the Amino-Acid Sequence of Sperm Whale Myoglobin and of Human Haemoglobin. Nature, 190: 670-672.

27. DeLorenzo, F., Goldberger, R.F., Steers, E. Jr., Givol, D., and Anfinsen, C.B., 1966. Purification and Properties of an Enzyme from Beef Liver which Catalyzes Sulfhydryl-Disulfide Interchange in Proteins. J. Biol. Chem., 241: 1562-1567.

28. Goldberger, R.F., Epstein, C.J., and Anfinsen, C.B., 1963. Acceleration of Reactivation of Reduced Bovine Pancreatic Ribonuclease by a Microsomal System from Rat Liver. J. Biol. Chem., 238: 628-635.

29. Goldberger, R.F., Epstein, C.J., and Anfinsen, C.B., 1964. Purification and Properties of a Microsomal Enzyme System Catalyzing the Reactivation of Reduced Ribonuclease and Lysozyme. J. Biol. Chem., 239: 1406-1410.

30. Reithel, F.J., 1963. The Dissociation and Association of Protein Structures, In ADVANCES IN PROTEIN CHEMISTRY, 18: 123-226, Academic Press, New York.

31. Changeux, J.P., 1964. Discussion on the Mechanism and Significance of Subunit Association, in Brookhaven Symp. Biol., 17: 174.

32. Subunit Structure of Proteins; Biochemical and Genetic Aspects, Brookhaven Symposia in Biology, Number 17, 1964.

33. Fincham, J.R.S., 1966. GENETIC COMPLEMENTATION, W.A. Benjamin, Inc., New York.

34. Monod, J., Changeux, J.P., and Jacob, F., 1963. Allosteric Proteins and Cellular Control Systems. J. Mol. Biol., 6: 306-329.

35. Changeux, J.P., 1964. Allosteric Interaction Interpreted in Terms of Quaternary Structure, in Brookhaven Symp. Biol., 17: 232-249.

36. Monod, J., Wyman, J., and Changeux, J.P., 1965. On the Nature of Allosteric Transitions: A Plausible Model. J. Mol. Biol., 12: 88-118.

37. Creighton, T.E., and Yanofsky, C., 1966. Association of the α and β_2 Subunits of the Tryptophan Synthetase of Escherichia coli. J. Biol. Chem., 241: 980-990.

38. Crawford, I.P., and Ito, J., 1964. Serine Deamination by the B Protein of Escherichia coli Tryptophan Synthethase. Proc. Natl. Acad. Sci., 51: 390-397.

39. Creighton, T.E., 1964. Discussion on the Mechanism and Significance of Subunit Association, in Brookhaven Symp. Biol., 17: 181-182.

40. Schwartz, D., 1962. Genetic Studies on Mutant Enzymes in Maize, II. On the Mode of Synthesis of the Hybrid Enzymes. Proc. Natl. Acad. Sci., 48: 750-756.

41. Allen, S.L., Misch, M.S., and Morrison, B.M., 1963. Genetic Control of an Acid Phosphatase in Tetrahymena: Formation

of a Hybrid Enzyme. Genetics, 48: 1635-1658.

42. Beckman, G.W., and Johnson, F.M., 1964. Variations in Larval Alkaline Phosphatases Controlled by Aph Alleles in Drosophila melanogaster. Genetics, 49: 829-835.

43. Loper, J.C., Grabner, M., Stahl. R.C., Hartman, Z., and Hartman, P.E., 1964. Genes and Proteins Involved in Histidine Biosynthesis in Salmonella, in Brookhaven Symp. in Biology, 17: 15-52.

44. Ahmed, A., Case, M.E., and Giles, N.H., 1964. The Nature of Complementation Among Mutants in the Histidine-3 Region of Neurospora crassa, in Brookhaven Symp. Biol., 17: 53-65.

45. Woodward, D.O., 1959. Enzyme Complementation in Vitro Between Adenylosuccinase-less Mutants of Neurospora crassa. Proc. Natl. Acad. Sci., 45: 846-850.

46. Fincham, J.R.S., and Coddington, A., 1963. Complementation at the Am Locus of Neurospora crassa: A Reaction Between Different Mutants Forms of Glutamate Dehydrogenase. J. Mol. Biol., 6: 361-373.

47. Suyama, Y., 1963. In Vitro Complementation in the Tryptophan Synthetase Mutants of Neurospora crassa. Biochem. Biophys. Res. Comm., 10: 144-149.

48. Loper, J.C., 1961. Enzyme Complementation in Mixed

Extracts of Mutants from the Salmonella Histidine B Locus, Proc. Natl. Acad. Sci., 47: 1440-1450.

49. Schlesinger, M.J., and Levinthal, C., 1963. Hybrid Protein Formation of E. coli Alkaline Phosphatase Leading to in Vitro Complementation. J. Mol. Biol., 7: 1-12.

50. Perrin, D., 1963. Complementation Between Products of the β-Galactosidase Structural Gene of Escherichia coli. Cold Spring Harb. Symp. Quant. Biol., 28: 529-532.

51. Kaplan, N.O., 1964. Lactate Dehydrogenase-Structure and Function, in Brookhaven Symp. in Biol., 17: 131-153.

52. Zipser, D., and Perrin, D., 1963. Complementation on Ribsomes. Cold Spring Harb. Symp. Quant. Biol., 28: 533-538.

53. Edgar, R.S., and Wood, W.B., 1966. Morphogenesis of Bacteriophage T_4 in Extracts of Mutant-Infected Cells. Proc. Natl. Acad. Sci. 55: 498-505.

54. Green, D.C., and Hechter, O., 1965. Assembly of Membrane Subunits. Proc. Natl. Acad. Sci., 53: 318-325.

55. Horowitz, N.H., and Fling, M., 1953. Genetic Determination of Tyrosinase Thermostability in Neurospora. Genetics, 38: 360-374.

56. Horowitz, N.H., and Fling, M., 1956. The Role of Genes in the Synthesis of Enzymes, in ENZYMES: UNITS OF

BIOLOGICAL STRUCTURE AND FUNCTION ed. by O.H. Gaebler, pp. 139-145, Academic Press, New York.

57. Horowitz, N.H., and Fling, M., 1956. Studies of Tyrosinase Production by a Heterocaryon of Neurospora. Proc. Natl. Acad. Sci., 42: 498-501.

58. Fling, M., Horowitz, N.H., and Heinemann, S.F., 1963. The Isolation and Properties of Crystalline Tyrosinase from Neurospora. J. Biol. Chem., 238: 2045-2053.

59. Sueoka, N., 1959. Genetic and Biochemical Studies of Tyrosinase in Neurospora, Ph.D. Thesis, California Institute of Technology.

60. Horowitz, N.H., Fling, M., MaCleod, H., and Watanabe, Y., 1961. Structural and Regulative Genes Controlling Tyrosinase Synthesis in Neurospora. Cold Spring Harb. Symp. Quant. Biol., 26: 233-238.

61. Horowitz, N.H., Fling, M., MaCleod, H., and Sueoka, N., 1961. A Genetic Study of Two New Structural Forms of Tyrosinase in Neurospora. Genetics, 46: 1015-1024.

62. Sussman, A.S., 1961. A Comparison of the Properties of Two Forms of Tyrosinase from Neurospora crassa. Arch. Biochem Biophys., 95: 407-415.

63. Horowitz, Fling, M., MaCleod, H.L., and Sueoka, N., 1960. Genetic Determination and Enzymatic Induction of

Tyrosinase in Neurospora, J. Mol. Biol., 2: 96-104.

64. Fox, A.S., and Gray, W.D., 1950. Immunogenetic and Biochemical Studies of Neurospora crassa: Differences in Tyrosinase Activity Between Mating Types of Strain 15300 (Albino-2). Proc. Natl. Acad. Sci., 36: 538-546.

65. Fox, A.S., and Burnett, J.B., 1959. The Genetics and Biochemistry of Tyrosinase in Neurespora crassa, in PIGMENT CELL BIOLOGY ed. by M. Gordon, pp. 249-277, Academic Press, New York.

66. Fox, A.S., Burnett, J.B., and Fuchs, M.S., 1959. Electrophoretically Separable Tyrosinases of Diverse Thermostabilities and Their Interconversion in Neurospora crassa. Genetics, 44: 510.

67. Fox, A.S., and Burnett, J.B., 1962. Tyrosinases of Diverse Thermostabilities and Their Interconversion in Neurospora crassa. Biochim. Biophys. Acta, 61: 108-120.

68. Fox, A.S., Burnett, J.B., and Fuchs, M.S., 1963. Tyrosinase as a Model for the Genetic Control of Protein Synthesis. Ann. N.Y. Acad. Sci., 100: 840-856.

69. Westergaard, M., and Mitchell, H.K., 1947. Neurospora V. A Synthetic Medium Favoring Sexual Reporduction. Am. J. Botany, 34: 573-577.

70. Horowitz, N.H., Personal Communication.

71. Pall, M. L., Horn, G., Fling, M., and Horowitz, N.H., 1965. A Simple Method for the Induction of High Levels of Tyrosinase Activity. Neurospora Newsletter, 8: 20.

72. Vogel, H.J., 1956. A Convenient Growth Medium for Neurospora (Medium N), Microbial Genetics Bulletin, 13: 42-43.

73. Mason, H.S., 1955. Comparative Biochemistry of the Phenolase Complex. Adv. in Enzymol., 16: 105-184.

74. Report of the Commission on Enzymes of the International Union of Biochemistry; Nomenclature of Enzymes and Coenzymes, published as Volume 13 of COMPREHENSIVE BIOCHEMISTRY ed. by M. Florkin and E.H. Stotz, 1964, Elsevier Publishing Company, Amsterdam.

75. Mason, H.S., 1948. The Chemistry of Melanin. III. Mechanism of the Oxidation of Dihydroxyphenylalanine by Tyrosinase. J. Biol. Chem., 172: 83-99.

76. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193: 265-275.

77. Kalckar, H.M., 1947. Differential Spectrophotometry of Purine Compounds by Means of Specific Enzymes. III. Studies of the Enzymes of Purine Metabolism. J. Biol. Chem., 167: 461-475.

78. Martin, R.G., and Ames, B.N., 1961. A Method for

Determining the Sedimentation Behavior of Enzymes: Application to Protein Mixtures. J. Biol. Chem., 236: 1372-1379.

79. Peterson, E. A., and Sober, H. A., 1962. Column Chromatography of Proteins: Substituted Celluloses, in METHODS IN ENZYMOLOGY, Volume 5, ed. by S. P. Colowick and N. O. Kaplan, pp. 3-27, Academic Press, New York.

80. Bock, R. M., and Ling, N., 1954. Devices for Gradient Elution in Chromatography. Anal. Chem., 26: 1543-1546.

81. Horowitz, N. H., and Shen, S. C., 1952. Neurospora Tyrosinase. J. Biol. Chem., 197: 513-520.

82. Jolley, R. L., Jr., and Mason, H. S., 1965. The Multiple Forms of Mushroom Tyrosinase. J. Biol. Chem., 240: PC1489-PC1491.

83. Margoliash, E. and Lustgarten, J., 1962. Interconversion of Horse Heart Cytochrome c Monomer and Polymers. J. Biol. Chem., 237: 3397-3405.