ENZYME-SUBSTRATE REACTIONS IN VERY HIGH MAGNETIC FIELDS. II

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ABSTRACT We have examined the effect of conditioning the enzyme trypsin in solution at pH 8.2 in a large magnetic field before determining its reactivity towards a synthetic substrate *N*-benzoyl DL-arginine *p*-nitroanilide (BAPA). This "pretreatment" was allowed to proceed for as long as 3⅝ hr in a magnetic field of 208 kgauss at temperatures 26 and 36°C. No effect on reactivity was observed when such pretreated enzyme solutions were compared with identical but untreated enzyme solutions. A single such reaction, allowed to proceed directly in a magnetic field of 220 kgauss for 9 min, similarly showed no difference in rate from its control.

INTRODUCTION

We have demonstrated previously (1) that in the case of all four enzymes examined, namely ribonuclease, polyphenoloxidase, peroxidase, and aldolase, exposure to magnetic fields of up to 170 kgauss for periods of time from 2 to 20 min gives no observable effect upon the rate of reaction of the enzymes with their substrates. In an earlier paper (2) similar results were found for succinate cytochrome *c* reductase in fields up to 50 kgauss. In those papers, we also examined the significant publications in this area of work and discussed our reasons for entering this field at this time. In light of these earlier works, we have felt that our investigation should be extended into two areas not yet examined by us.

The first of these is the use of long periods of exposure to the magnetic field by the system under investigation. This has very recently been made possible by a redesigning of the circuit energizing the largest magnet at present available in the National Magnet Laboratory (Massachusetts Institute of Technology, Cambridge, Mass.) making it capable of steady DC fields of up to 220 kgauss for up to 2 hr.

The second involves the preconditioning of the enzyme in the magnetic field, in the absence of its substrate, before assaying the enzyme in the absence of an applied magnetic field. These extensions would bring our investigation more closely in line with the investigations of Smith,[1] Cook, and Smith (3), Wiley (4), and Akoyunoglou

[1] M. J. Smith. Private communication.

(5), all of whom have observed an apparent effect of exposure to fields of up to 20 kgauss for extended periods of time.

To this end, that a comparison may be made, we chose to work with trypsin and its synthetic substrate *N*-bensoyl DL-arginine *p*-nitroanilide (BAPA) which were used by Smith and Cook (3). Like these authors we were able to preexpose the enzyme, before assay, to the field for more than 3 hr, but unlike these authors our fields were 208 kgauss as compared to 8 kgauss.

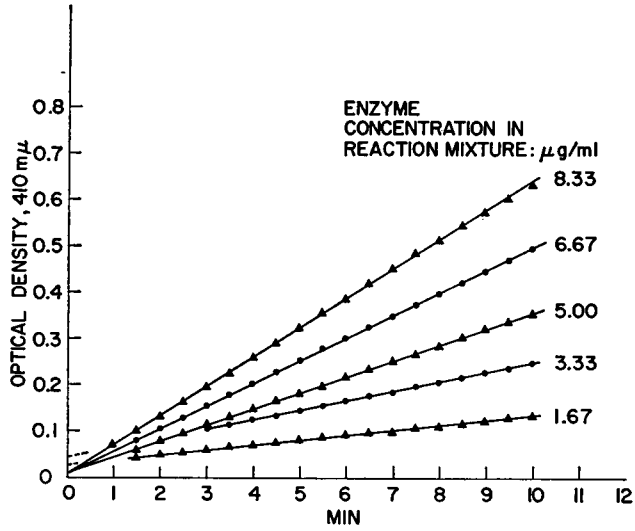

FIGURE 1 Rates of trypsin—BAPA reactions at various enzyme concentrations at 26°C.

MATERIALS AND METHODS

The trypsin used was two times crystallized, lyophilized, dialyzed salt-free, coded TRL6FA, and obtained from Worthington Biochemical Corporation (Freehold, N. J.). Its synthetic substrate BAPA was obtained from Nutritional Biochemical Corporation (Cleveland, Ohio) and used without further purification. The method of assay was that described by Erlanger et al. (16) as method II, with slight modifications to allow for transfer of larger volumes of enzyme solution to the substrate and to allow for following the reaction over a period of time. This method is briefly described as follows.

5 mg of trypsin were dissolved in 100 ml 10^{-3} N HCl and kept refrigerated when not in use. Dilutions of this were made when required. The substrate solution was made up as 10^{-3} M BAPA in a 0.05 M Tris buffer, pH 8.2, being 0.02 M in $CaCl_2$. The BAPA was completely dissolved in 2 ml dimethyl sulfoxide before adding the buffer. The reaction was started by adding 1 ml of enzyme solution to 5 ml substrate solution, both of which had been kept at 26°C in a thermostated bath. The reaction results in the production of *p*-nitroaniline which absorbs at 410 mμ and was followed by transmittance measurements at 410 mμ in a Beckman DU spectrophotometer with a thermostated (26°C) cell compartment (Beckman Instruments, Inc., Fullerton, Calif.)

Fig. 1 shows the course of a series of trypsin reactions at various enzyme concentrations, and Fig. 2 shows the dependence of the rate of the reaction on enzyme concentration. In this

latter figure, similar data obtained by Erlanger et al. (16) were included. The procedure adopted for experimental runs with the magnet was somewhat different from that described above. A stock solution of trypsin, exactly six times the concentration to be used in the hydrolytic reaction, was divided into two parts, one part being placed in the field, the other in the thermostated bath. The temperature control of the magnet sample has been described in an earlier publication (1).

At the end of a prescribed period of exposure to the field, the magnet sample was withdrawn from the field and rapidly placed in the thermostated bath. A 1 ml aliquot was withdrawn and mixed with 5 ml of 10^{-3} N HCl as diluent, already equilibrated at the temperature of the bath, the mixing being complete at 1 min after withdrawal from the field. The stock

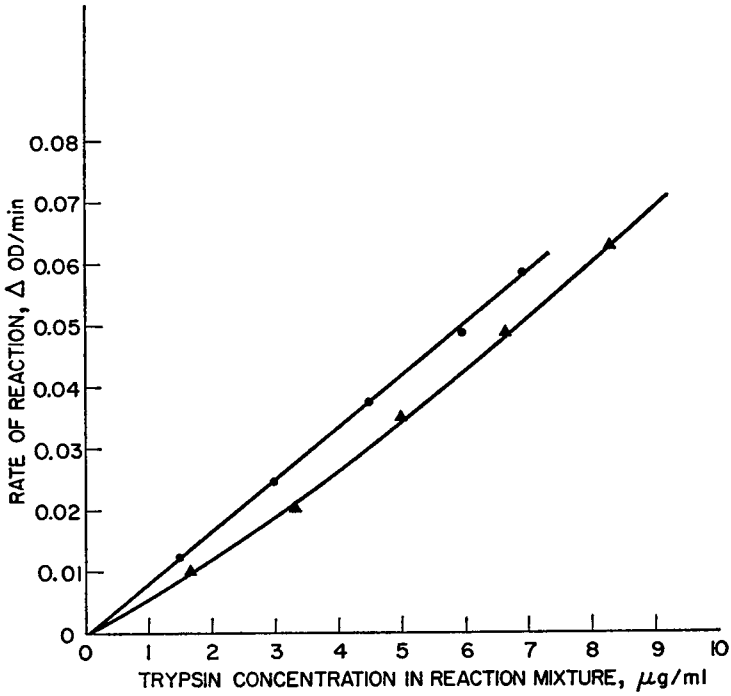

FIGURE 2 Dependence of trypsin—BAPA reaction rate on enzyme concentration at 26°C. ▲, our results. ●, results of Erlanger et al. (6).

enzyme solution was immediately replaced in the field. After a 1 min interval, the stock enzyme control solution was diluted in a similar way. 1 min later, a 1 ml aliquot of the diluted, field-exposed, enzyme solution was mixed with 5 ml of the thermostated substrate solution, followed 40 sec later by a similar transfer for the diluted control enzyme solution. We thus had two reactions started 40 sec apart which, aside from any possible effect of the exposure to the magnetic field, should take exactly similar courses. These reactions were compared alternately against a blank, after transfer to cuvettes, in a Carey recording spectrophotometer (Applied Physics Corporation, Monrovia, Cal.) at room temperature.

This procedure was repeated at intervals of time, using the same diluted enzyme solutions, which had been kept thermostated, until a second aliquot was obtained from both the stock enzyme solution, still being exposed to the field, and the stock enzyme control solution. The

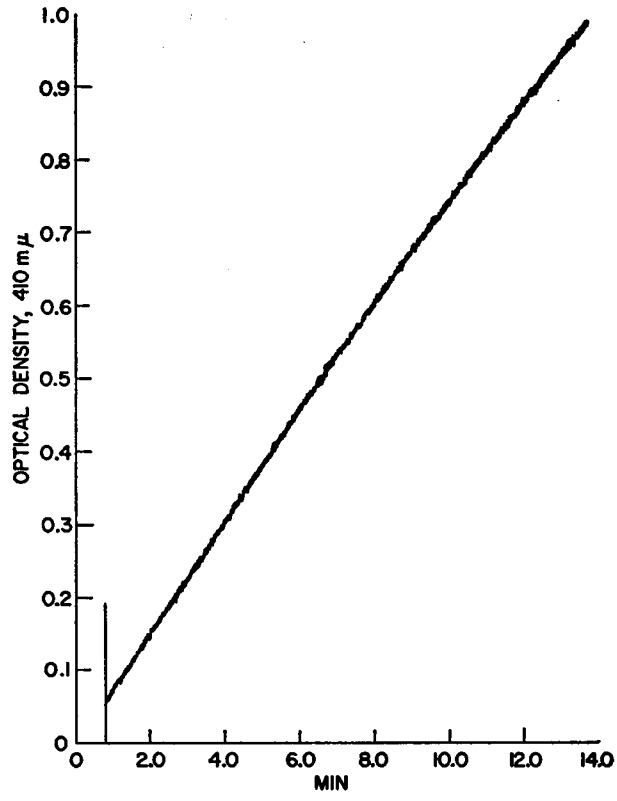

FIGURE 3 Typical record of course of trypsin—BAPA reaction kept at 26°C for 17 min before observation in a Carey recording spectrophotometer. Enzyme concentration in reaction mixture, 8.7 μg/ml.

TABLE I

INACTIVATION OF TRYPSIN AT 26.0°C

Concentration of enzyme in the reaction mixture, 8.7 g/ml

Run number	Time at 26°C	Rate of reaction
	min	*Δ OD/min*
1	0	0.0740
2	17	0.0765*
3	32	0.0721
4	60	0.0716
4A	76	0.0701

* See Fig. 3.

dilution and initiation of reaction were repeated as above until a third aliquot was removed from the field-exposed stock enzyme solution.

In this way, identically treated enzyme solutions, magnet run, and control were compared, except for (*a*) increasing periods subsequent to field exposure before assay and (*b*) increasing

exposure to the field for up to 3⅔ hr. This procedure allowed us to examine the decay or delayed activation of any field effect. In order to keep the magnet on for such long periods, these experiments were run at a field of 208 kgauss.

EXPERIMENTAL RESULTS

As a preliminary to the magnet runs, several reactions were followed, with zero applied field, by keeping the enzyme solution, concentration 52 μg/ml, and the sub-

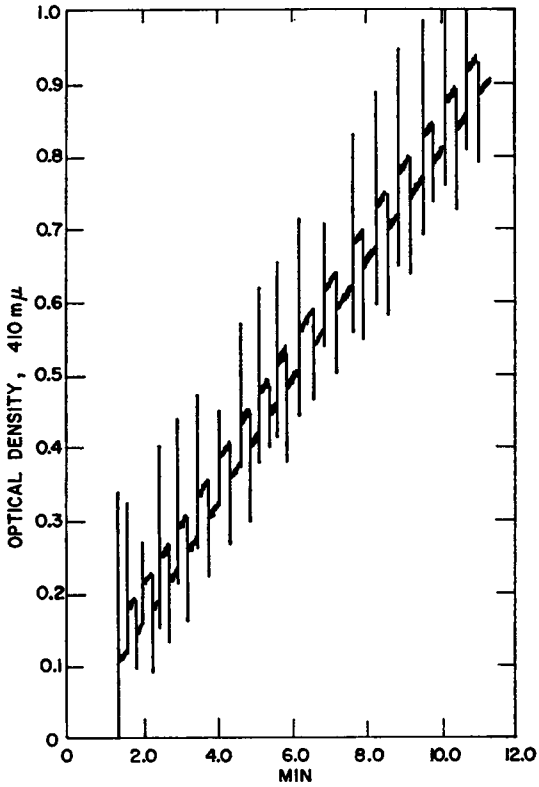

FIGURE 4 Typical record of comparison of a field-exposed trypsin—BAPA reaction (upper curve) with its control (lower curve) at 26°C. Exposure time, 65 min. Enzyme concentration in reaction mixture, 8.7 μg/ml. Field strength, 208 kgauss.

strate solution at 26°C, and starting the reaction in the unmodified manner described above. Typical data obtained are shown in Fig. 3, where the optical density of the reacting system was followed as a function of time at 410 mμ. Successive reactions were carried out using the same component solutions, kept at 26°C, as a means of determining the accuracy of the assay and the extent of inactivation at this temperature. The results are given in Table I.

These determinations indicate a probable error in the measurement of not more than 2% and a slow inactivation at this temperature of close to 5% in an hour.

Fig. 4 shows a typical experimental result of comparing the rate of reaction of a field-exposed enzyme with its substrate to that of its control at 26°C. In this case the

exposure time was 65 min and the magnet reaction (M) was started 3 min after dilution of the stock enzyme solution. This was followed 40 sec later by the initiation of the control reaction (NM). In subsequent reactions, we alternated between starting the magnet run reaction first and starting the control reaction first.

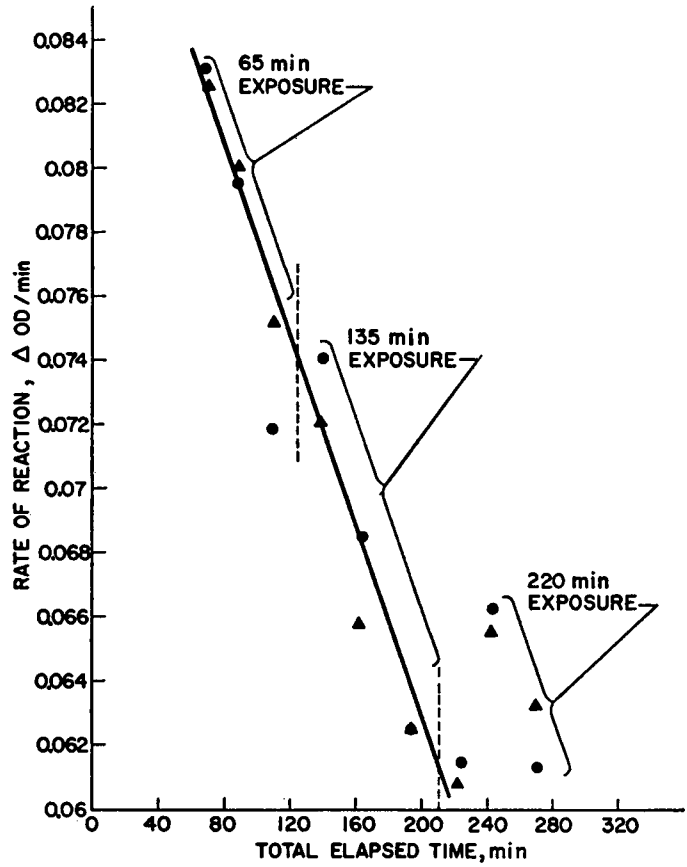

FIGURE 5 Comparison of reaction rates of field-exposed trypsin—BAPA reactions (●) with their controls (▲) at 26°C. Three exposure times. Total elapsed time from beginning of shortest exposure. Enzyme concentration in reaction mixture, 8.5 μg/ml. Field strength, 208 kgauss.

Data for a series of such reactions at 26°C are summarized in Fig. 5. It is clear that the differences between the rates of the magnet run reactions and those of their control reaction show no trend either as a function of the time of exposure of the enzyme to the field or as a function of the time lapsed since removal from the field. It is worth noting that even though some uncertainty appears in the absolute rates determined after long exposure to the field, the comparative rates of magnet runs

and their controls lie well within experimental error, which is calculated from this data to be ± 1 %.

Similar data obtained at 36°C are shown in Fig. 6. Further data at this temperature obtained at a different time with a different enzyme solution which had been kept at approximately 5°C for some 36 hr before use are given in Table II.

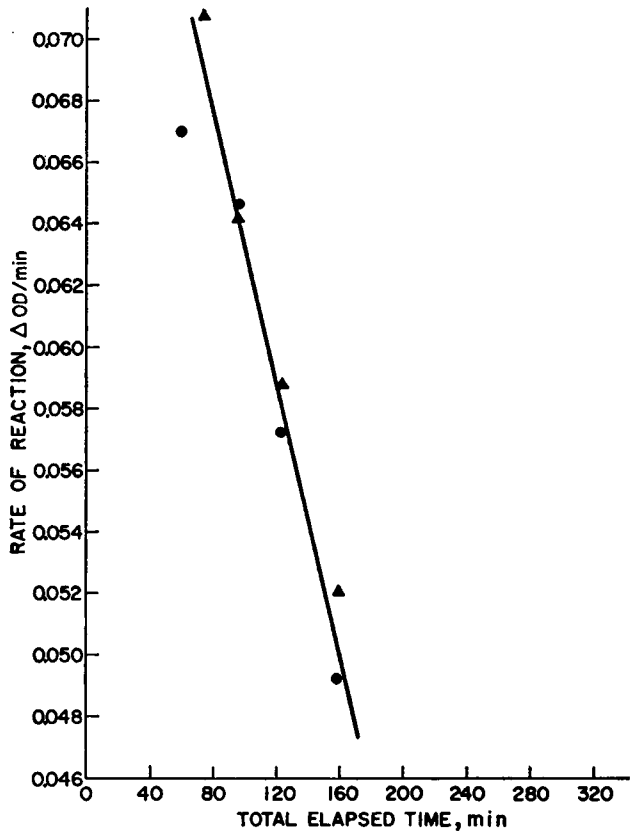

FIGURE 6 Comparison of reaction rates of field-exposed trypsin—BAPA reactions (●) with their controls (▲) at 36°C. Exposure time, 57 minutes. Total elapsed time from beginning of exposure. Enzyme concentration in reaction mixture, 5.86 μg/ml. Field strength, 208 kgauss.

In order to make these experiments more complete and to extend the type of experiment discussed previously (1, 2), a reaction with this same enzyme preparation and substrate was carried out in the full field of the magnet (220 kgauss).

The procedure adopted was the following.

The enzyme stock solution was diluted six times with 10^{-3} N HCl to give a concentration of 35 μg/ml and thermostated, as was the substrate, at 36°C. The reaction

TABLE II

COMPARISON OF THE REACTION RATES OF SOME FIELD-EXPOSED (M) TRYPSIN WITH ITS CONTROL (NM) AT 36°C

Enzyme concentration in the reaction mixture, 5.84 g/ml

Run number	Type	Field exposure	Time after dilution	Rate	Difference
		min	*min*	Δ *OD/min*	±%
34	M	67	2	0.0712	0.3
	NM	67	2	0.0708	
35	M	67	22	0.0695	0.4
	NM	67	22	0.0690	
36	M	67	41	0.0788	0.8
	NM	67	41	0.0800	
37	M	133	2	0.0732	1.4
	NM	133	2	0.0712	
38	M	133	23	0.0692	0.4
	NM	133	23	0.0698	
39	M	180	7	0.0728	0.7
	NM	180	7	0.0738	
40	M	180	28	0.0655	1.5
	NM	180	28	0.0675	

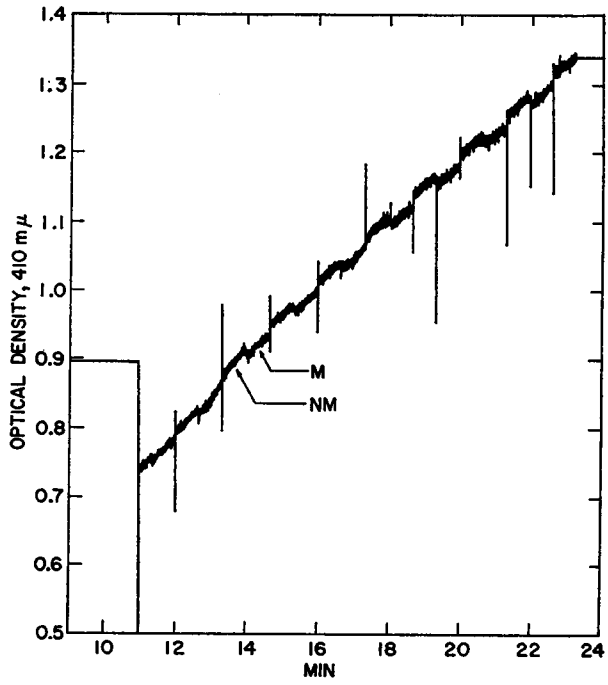

FIGURE 7 Comparison of trypsin—BAPA reaction course after running for 9 min in a field of 220 kgauss (M) with its control (NM) at 36°C. Enzyme concentration in reaction mixture, 5.84 μg/ml.

was started by adding 2 ml of the enzyme solution to 10 ml of the substrate solution while thermostated. One-half of this reaction mixture (enzyme concentration, 5.84 μg/ml) was placed in a Pyrex glass tube and inserted into the field (1) (220 kgauss) while the remainder, acting as a control, was placed back in the thermostat bath. The reactions were allowed to proceed for 9 min before being removed, placed in cuvettes, and compared alternately against a blank in the Carey recording spectrometer at 410 mμ.

The result is shown in Fig. 7, and it is clear that within experimental error, the courses of the two reactions are the same.

CONCLUSION

It is apparent from the results presented here, that a pretreatment of the enzyme trypsin in large magnetic fields for prolonged periods of time has no more effect on its rate of reaction with the substrate BAPA than exposure of the reaction mixture to the field, both being equal to zero. The pretreatment exposure time of 3 hr 40 min is closely similar to the times used by Cook and Smith (3) and Smith,[1] and the conditions were identical with one set of conditions used by them (3). We have differed from them only in using much larger fields than they did (8 and 13 kgauss).

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