ENZYME-SUBSTRATE REACTIONS IN VERY HIGH MAGNETIC FIELDS. I

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ABSTRACT The availability of very high magnetic fields of up to 170,000 gauss made it worthwhile to pursue the search for a critical change in the rate of four enzyme substrate reactions. The four enzymes were ribonuclease, polyphenol oxidase, peroxidase, and aldolase. The experiments showed that, to within $\pm 3\%$, no detectable change was observable in the rate of reaction of any of the systems for periods of exposure to the magnetic field of up to 20 min.

INTRODUCTION

In an earlier paper by Maling, Weissbluth, and Jacobs (1), the reasons for venturing into the controversial field of biomagnetics were given and discussed in terms of the theoretical work of Little (2) on superconductivity and conducting macromolecules. Further reference should be made to the work of Cook and Smith (3), of Smith,[1] and of Wiley et al. (4). In all of these, an effect was found, apparently due to the application of a magnetic field to the enzyme, in the absence of its substrate.

In the first of these (3), fields of 8 kilogauss applied for up to 3 hr are reported to have given an average increase in trypsin activity of 11% but not greater than 23% above that of the unexposed enzyme at pH 3.0. In the second,[1] fields of 13 kgauss applied for up to 4 hr similarly are reported as having given an average increase of 12% (but not more than 24%) in trypsin activity for the enzyme at pH 3.0, but an average decrease of 7.5% (but not greater than 9%) for the enzyme at pH 8.0. In the third work (4), fields of 5 kgauss, applied for 17 hr, are reported to give a partial restoration of trypsin activity of up to 12% at pH 3.0 after inhibition by egg white.

The work of Maling et al. (1) reports that there was no observable effect on either ribonuclease or succinate-cytochrome *c* reductase up to fields of approximately 50 kgauss. However, Little's calculations do not predict the field strength at which the hypothesized superconductivity of the macromolecule is lost, unless one can

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

extrapolate from a law established for metallic superconductors, and applicable to temperatures of about 1° to 10°K, to macromolecules at 2000°K. We only know that the critical fields are likely to be very high, and if Little is correct, the effect dramatic.

The availability of magnetic fields of the order of 170 kgauss has again made it reasonable to look for a biomagnetic phenomenon, especially since such a field

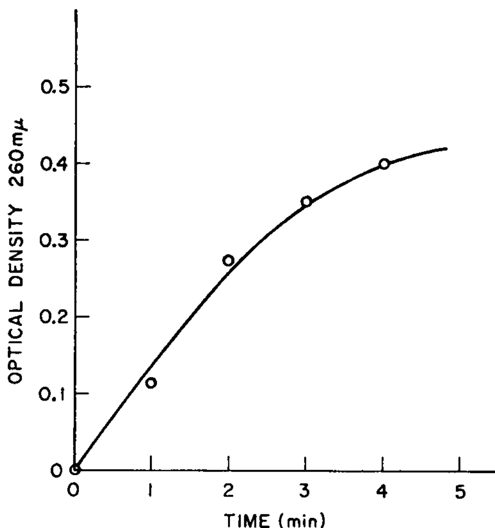

FIGURE 1 RNase reaction rate at 37°C. Enzyme concentration 6 μg/ml before adding substrate.

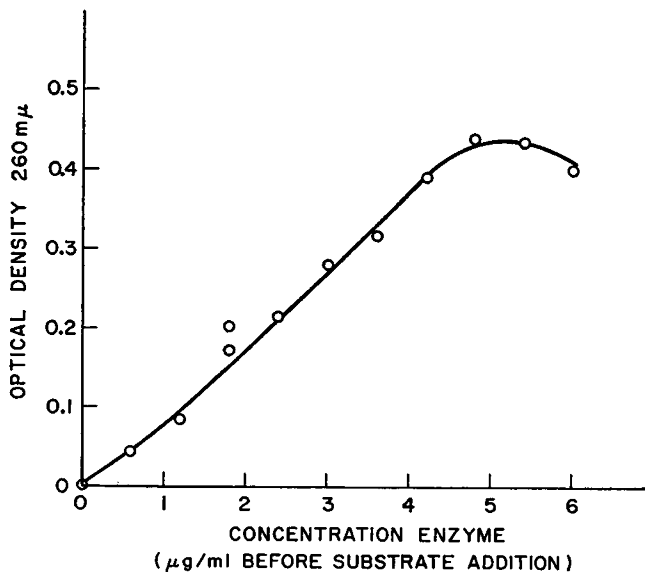

FIGURE 2 Effect of RNase concentration on rate of reaction (4 min assay) at 37°C.

strength already approaches the critical field predicted by the extrapolation mentioned above.

Four enzyme-substrate systems were chosen for examination in order to cover a variety of properties and characteristics, the pertinent ones listed as follows: (*a*)

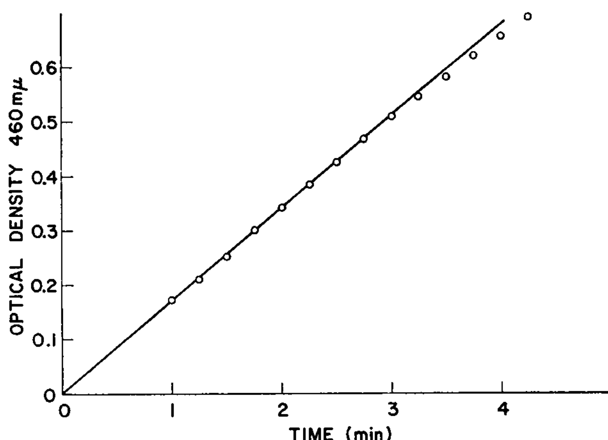

FIGURE 3 Pase reaction rate at 25°C. Enzyme concentration 1.33×10^{-5} mg/ml in reaction mixture.

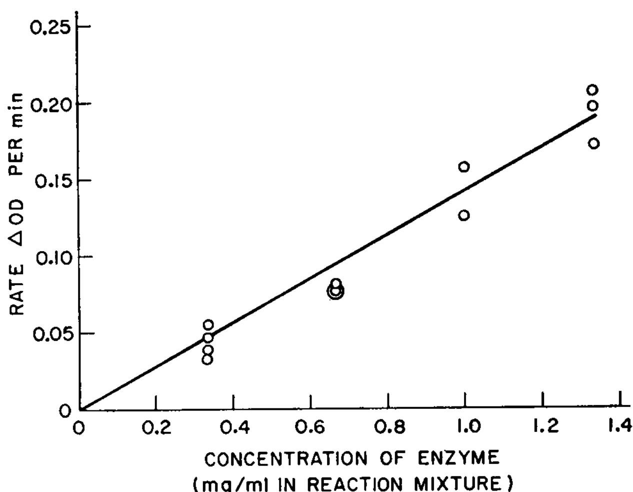

FIGURE 4 Effect of Pase concentration on rate of reaction at 25°C.

ribonuclease—for continuity of the earlier experiments (1); and for its use of a macromolecular substrate, (*b*) peroxidase—an iron-porphyrin enzyme containing a paramagnetic ion; it is very unspecific in both its substrate and in its source of oxygen; and it probably involves a free radical reactive species; (*c*) polyphenol

oxidase—it contains a nonparamagnetic ion, copper; it is unspecific in its substrate but highly specific with regard to its source of oxygen; and it gives rise to a polymerized product by a probable free radical mechanism; and (*d*) aldolase—it shows a highly specific reactivity towards a relatively simple molecule; it is free from any metallic ion as an intrinsic part of the molecule.

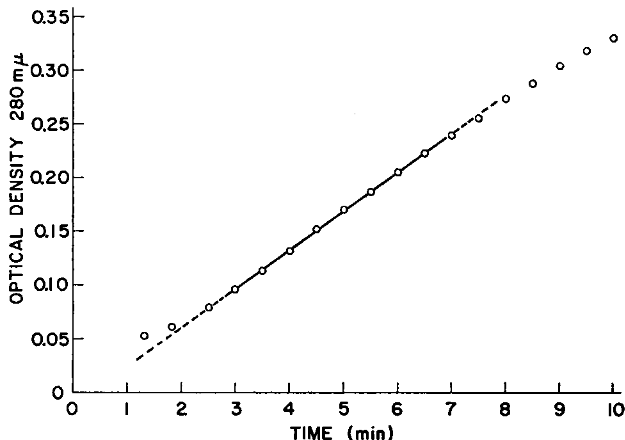

FIGURE 5 Tase reaction rate at 27°C. Enzyme concentration 5.0×10^{-2} mg/ml before adding substrate.

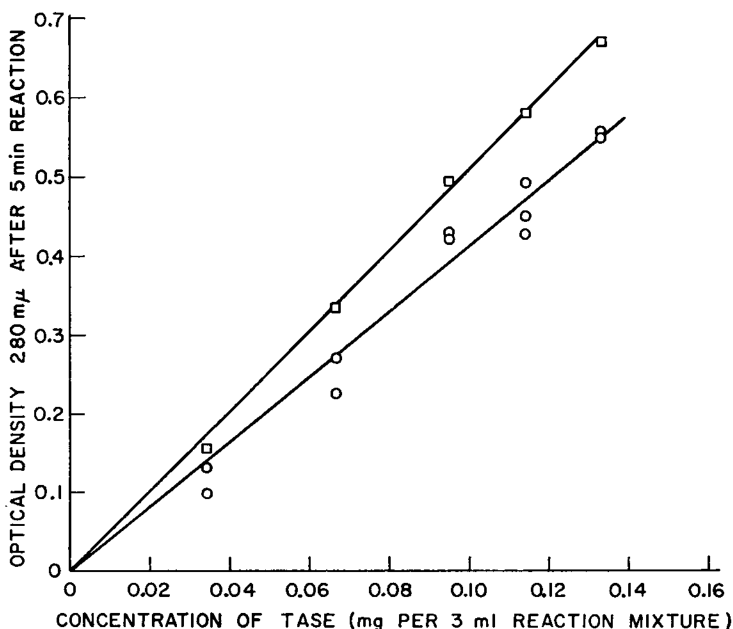

FIGURE 6 Effect of Tase concentration on rate of reaction at 27°C. □, reaction allowed to go to completion; ○, reaction stopped after 5 min by addition of 0.5 ml. 10^{-2} M cyanide ion.

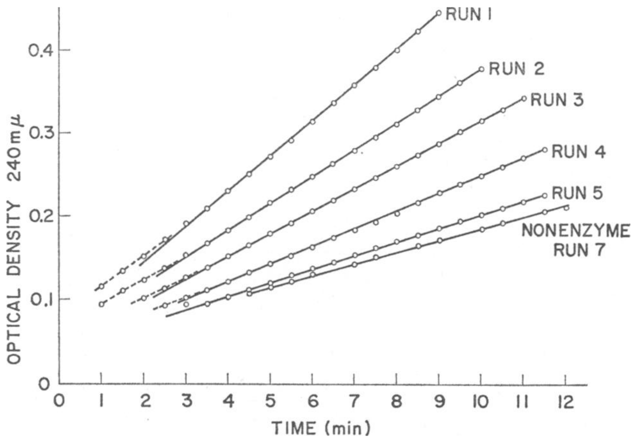

FIGURE 7 Aase reaction rates at 27°C for series of enzyme concentrations. Runs 1 through 5 are respectively 6.05, 4.84, 3.63, 2.42, and 1.21 $\times 10^{-3}$ mg/ml in the reaction mixture.

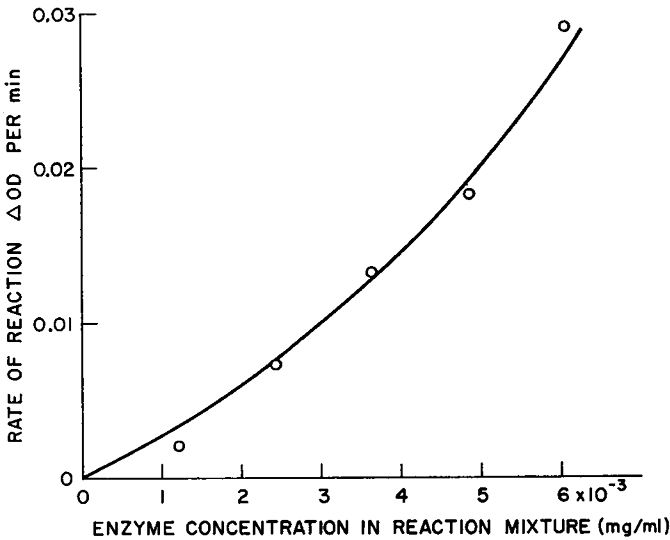

FIGURE 8 Effect of Aase concentration on rate of reaction at 27°C.

MATERIALS AND METHODS

The enzyme-substrate systems chosen were characterized in the following ways:

1. Ribonuclease (RNase). Bovine pancreas RNase was obtained from Worthington[2] as a lyophilized, phosphate-free powder, and as substrate we used a yeast ribonucleic acid (RNA).[3] The method of assay was that of Kalnitsky, Hummel, and Dierks (5) and briefly is as follows:

[2] Worthington Biochemical Corporation, Freehold, N. J.
[3] Schwartz Bio Research Inc., Orangeburg, N. Y.

(*a*) 6 mg of enzyme was dissolved in 100 ml of water as stock solution and, when required for use, was diluted 10 times with a 0.1 M acetate buffer pH 5.0.

(*b*) 1.0 ml of enzyme solution was allowed to incubate at 37°C with 1.0 ml of a 1.0% solution of RNA for 4 min.

(*c*) The reaction was stopped by addition of 1.0 ml of a 0.75% solution of uranyl acetate in 25% perchloric acid and cooled in ice.

(*d*) The precipitate of unreacted RNA and enzyme was filtered off by two passings through Whatman No. 1 paper and the filtrate of oligonucleotides diluted 25 times.

(*e*) The optical density was measured in a Beckman DU 2 at 260 mμ.

Assayed in this way (Fig. 1) the enzyme sample originally gave an activity of 1670 units/mg. Fig. 2 shows the dependence of reaction rate on enzyme concentration.

2. *Horseradish Peroxidase* (*Pase*). This enzyme was obtained[2] as a lyophilized powder and the substrate, the aniline derivative σ-dianisidine, was obtained from the same source. The reaction involved is simply the catalytic oxidation of the σ-dianisidine by hydrogen peroxide producing a colored dye. The assay method as described by Worthington gave a specific activity of 1120 units/mg (Fig. 3). Fig. 4 shows the effect of Pase concentration on reaction rate.

In practice, we used a modified version of the above assay method, in order to transfer larger volumes of reactants:

(*a*) A stock solution of Pase was made up of 10 mg dissolved in 10 ml water. In use, this solution was diluted in the order of $3 \rightarrow 6 \times 10^4$ times, just prior to use.

(*b*) The substrate reagent was made up with 1.5 ml of a 0.3% hydrogen peroxide solution with 0.6 ml of a 1.0% σ-dianisidine solution in methanol, the whole made up to 100 ml with a 0.01 M phosphate buffer pH 5.8.

(*c*) Equal volumes of enzyme solution and substrate reagent were mixed and allowed to react at 25°C. OD measurements are taken at 460 mμ at half-minute intervals for about 5–10 min.

Comparison of the unmodified with the modified Worthington assay showed a ratio of 1.7:1.0 in rate of reaction.

3. *Polyphenol Oxidase* (*Tyrosinase, Tase*). This enzyme was obtained[2] as a lyophilized powder from a mushroom source, and L-tyrosine was used as substrate. The work of Raper (6) shows the reaction to be a complex one, involving several steps initiated by the catalytic oxidation by molecular oxygen of the tyrosine to 3,4-dihydroxyphenyl alanine, the successive oxidation of the 3,4-dihydroxyphenyl alanine to the corresponding σ-quinone, and the final polymerization of the quinone to melanin.

The assay method is described by Worthington and is as follows:

(*a*) A stock solution of enzyme is prepared by dissolving 20 mg in 10 ml of a 0.1 M phosphate buffer pH 7.0. This solution is diluted, just prior to use, to about 5 mg/100 ml with water, and oxygenated for 5 min at room temperature.

(*b*) The substrate is a 1.0×10^{-3} M L-tyrosine solution in water, and the reaction mixture is buffered with a 0.5 M phosphate buffer at pH 6.5.

(*c*) Equal volumes of enzyme and substrate are mixed at 25°C and OD measurements at 280 mμ taken at half-minute intervals for approximately 10 min.

With this assay method, our enzyme sample gave an activity of 730 units/mg (Fig. 5). Fig. 6 shows the dependence of reaction rate on enzyme concentration.

4. *Aldolase* (*Aase*). This enzyme, prepared from a rabbit muscle source, was obtained from Worthington[2] as a crystalline suspension in 0.52 saturated ammonium sulfate,

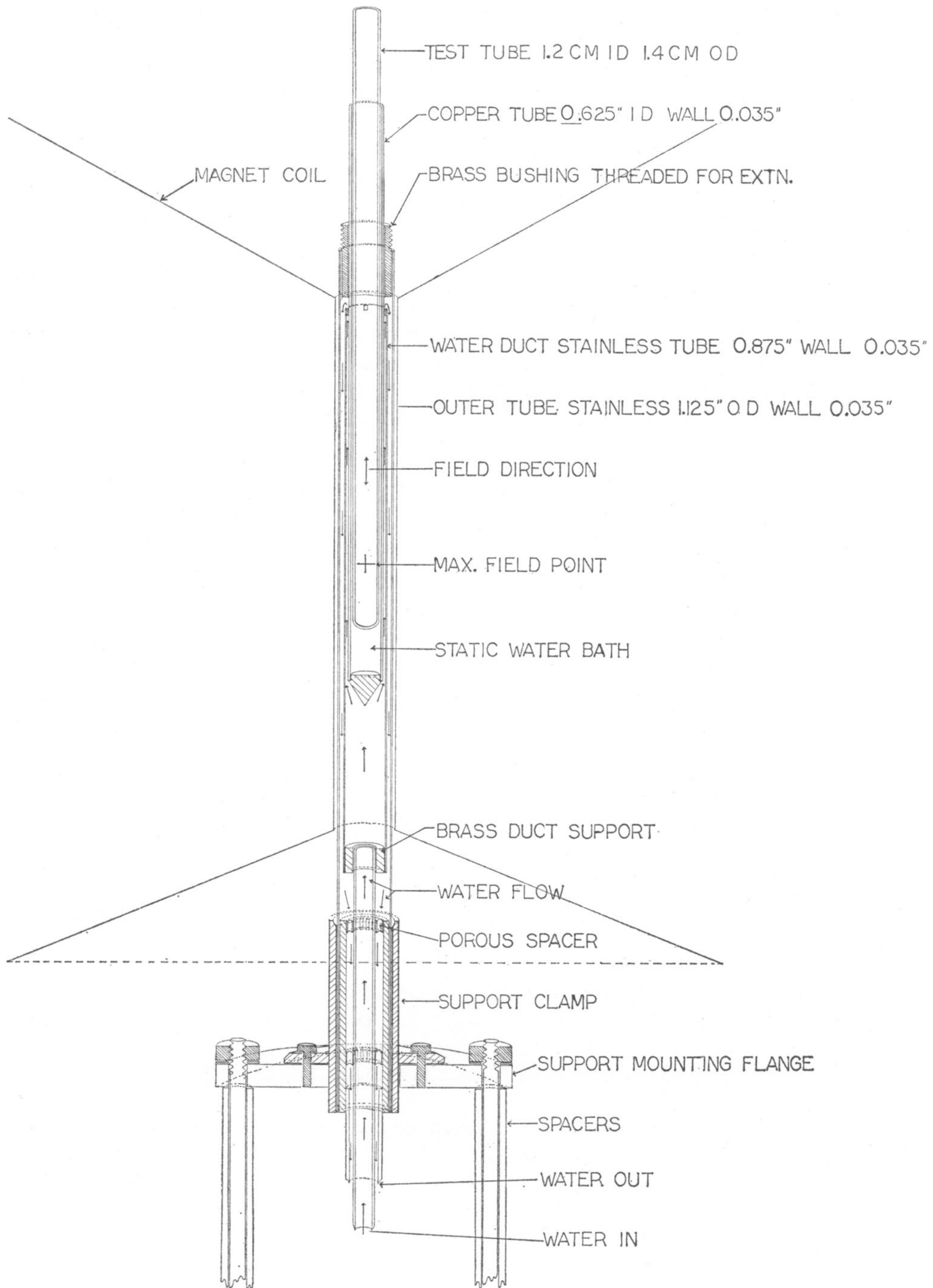

FIGURE 9 **Constant temperature unit for magnet runs.**

pH 7.8. It is capable of catalyzing the cleavage of fructose 1,6-diphosphate (FDP) into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (GAP). The assay method used was a Worthington modification of a hydrazine assay (7) in which hydrazine is allowed to react with the GAP giving a hydrazone which absorbs at 240 mμ. In practice, we changed the concentrations of the reactants in such a way that larger volumes of enzyme could be transferred while final reacting concentrations remained the same:

(*a*) The enzyme was diluted with water just prior to use to an appropriate activity level. Our FDP sample was assayed at 62.8% and therefore a substrate concentration of 8.75 g/liter was used. The hydrazine reagent was made up as 4.52×10^{-3} M hydrazine sulfate in 10^{-4} M EDTA pH 7.5.

(*b*) 1.0 ml of enzyme solution was added to 1.0 ml substrate solution plus 2.0 ml hydrazine solution, and transmittance measurements at 240 mμ were made for at least 10 min. It was necessary to correct such measurements by subtracting a nonenzymatic rate of FDP cleavage. Assayed in this way (Fig. 7) our enzyme gave an activity of 3.6 units/mg.

Fig. 8 shows the dependence of reaction rate on enzyme concentration.

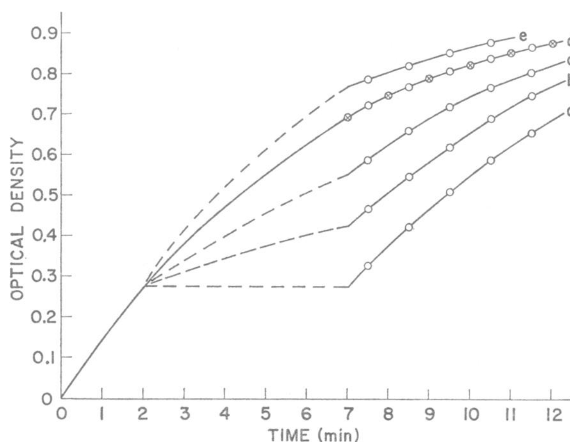

FIGURE 10 Course of hypothetical reaction and effects of magnetic field. ○, magnet run; ⊗, control run.

High Field Experiments

All of the experiments in high magnetic fields described here were carried out in Bitter solenoids designed to carry extremely high currents and to dissipate large amounts of heat by high rates of flow of cooling water. These magnets were made available to us by courtesy of the National Magnet Laboratories of Massachusetts Institute of Technology, Cambridge, Mass.

We will describe briefly only one of two such magnets used although the RNase experiments were carried out in a magnet of slightly lesser capabilities. The magnet, designated 1E, was rated at 5 MW and was capable of a maximum field of 175 kgauss when 22.2×10^3 amp were passed. The magnet was built essentially as a solenoid with a series of copper plates in which an ordered array of holes was drilled. These plates were stacked with interleaved dielectrics in such a way that current could flow through them in a predetermined helical path while cooling water was forced under high pressure through the paths created by the holes. Through the center of the plates was a hole of bore 1¼ inches and 11⅛ inches

long, in which the resultant field was concentrated. The homogeneity of the field was such that there was a drop of 5% of the magnet field in a distance 7 cm along the bore from the center.

All enzyme reactions in the magnetic field (referred to as "magnet runs") were carried out in pyrex glass tubes (300 × 15 mm) which were placed in a double walled, stainless steel, thermostated tube (Fig. 9). This, in turn, was located in the bore of the magnet so that the reaction mixture was located around the center of the magnet. Water, whose temperature

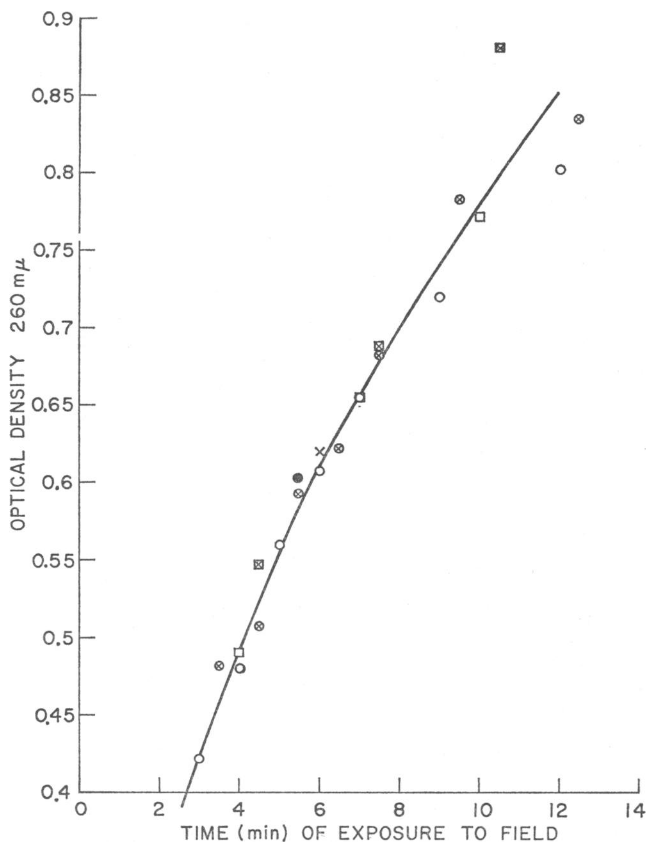

FIGURE 11 RNase assay at 37°C. Effect of magnetic fields up to 150,000 gauss. Simulated runs (zero field): ○, magnet; ⊗, control. Magnet runs at 150,000 gauss; □, magnet; ⊠, control. Magnet run at 100,000 gauss: ●, magnet; ×, control. Enzyme concentration 1.5 μg/ml before adding substrate.

was controlled in a constant-temperature bath, was circulated through the "magnet tube." The temperature differential between tube and bath was no greater than 0.05°C.

In order to eliminate differences in composition between magnet runs and control runs (reactions carried out simultaneously under exactly similar conditions but in the absence of an applied magnetic field), we adopted a procedure which was designed to accentuate possible small effects produced by the field.

A given system was mixed from reactants kept in the constant-temperature bath, and

rapidly transferred to two identical pyrex tubes kept in the bath. Soon thereafter one such tube was placed in the field (magnet run) while the other was handled in exactly the same way but replaced in the bath (control run). After the predetermined exposure to the field, both solutions were transferred to cuvettes and alternately compared to the blank, at the chosen wavelength, in a Beckman DU 2 spectrophotometer. Fig. 10 shows the kind of results to be expected from a hypothetical reaction, the course of which is of the same kind as those observed. In Fig. 10, the reaction is allowed to proceed for 2 min before separation into control and magnet runs. The exposure to the magnetic field is 5 min and curves *a*, *b*,

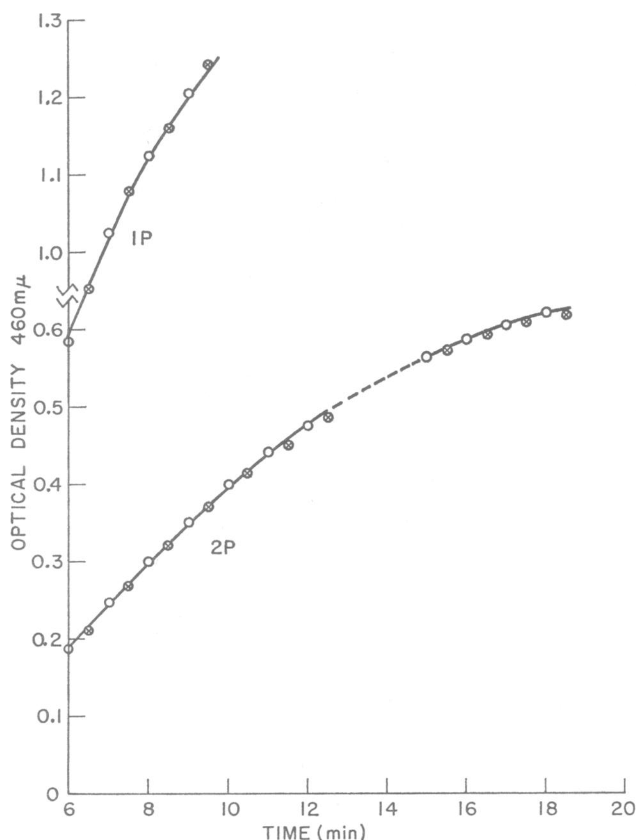

FIGURE 12 Pase reaction rate at 25°C. Simulated runs with 3 min exposure. ○, Magnet; ⊗, control. Enzyme concentrations: 1P, 3×10^{-5} mg/ml; 2P, 1.5×10^{-5} mg/ml in reaction mixture. Reaction placed in magnet at 2 min.

and *c* show the course of the magnet run assuming that the field stops the reaction (*a*), or retards it (*b*, *c*). Curve *d* shows the magnet run unaffected by the field and curve *e* an acceleration produced by the field. It is clear that as little as a 10% change in rate produced by the field will show up as a marked displacement in time of the two reactions, the displacement increasing with increasing exposure to the field.

A variation of this procedure was necessary in the case of RNase since the assay method necessitated the stopping of the reaction before spectrophotometric examination. Rather

than keeping the field exposure time constant and following the reactions subsequent to exposure, it was more convenient simply to subject the magnet runs to increasing times of exposure to a fixed field before stopping the reaction and comparing it to its control run. The resulting behavior is subject to the same analysis described for the hypothetical case.

EXPERIMENTAL RESULTS

(*a*) *Ribonuclease.* The results for RNase are shown in Fig. 11. The maximum field available at the time of the experiment was 150 kgauss. One run was

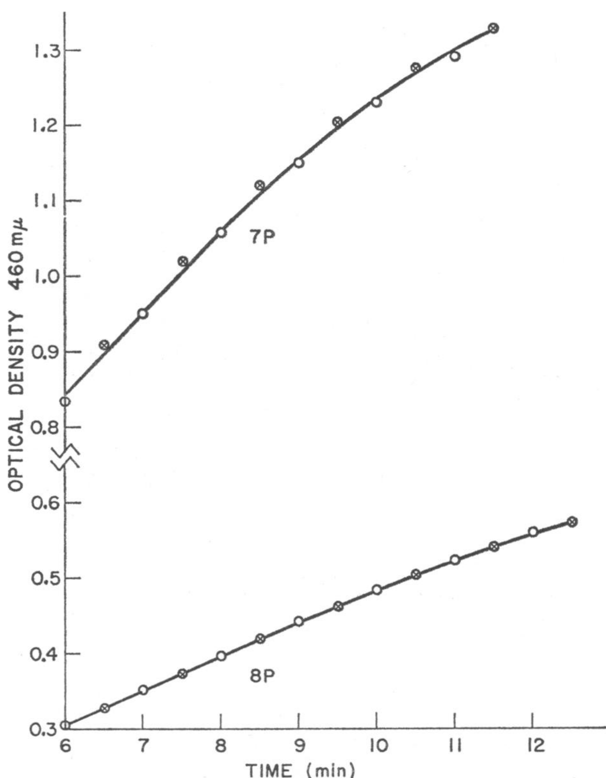

FIGURE 13 Pase reaction rate at 25°C. Effect of magnetic field of 170,000 gauss, 3 min exposure. ○, Magnet; ⊗, control. Enzyme concentrations: 7P, 3.0×10^{-6} mg/ml; 8P, 7.5×10^{-6} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

carried out at 100 kgauss. The points corresponding to "simulated runs" were carried out in exactly the manner described above but the magnet current was zero.

It is clear that the curve drawn satisfies all the points up to the maximum exposure to field of 10 min. It will be noted that, of the 10 min run, it is the control run that lies off the curve while the magnet run lies essentially on the curve. From this data we would estimate a probable deviation from the curve of not more than 0.02 in optical density, or $\pm 3\%$.

(*b*) *Peroxidase*. The results for Pase are shown in Figs. 12–15. Simulated runs have the same meaning as described under RNase, i.e. magnet runs with zero field and their corresponding control runs. Such runs for 3 and 10 min exposure are shown in Figs. 12 and 14 at various enzyme concentrations, while magnet runs for 3, 10, and 20 min exposure at full field (170 kgauss) are shown in Figs. 13 and 14. In Fig. 15, we show a 10 min magnet run at 85 kgauss and a 10 min magnet run carried out in a field where the gradient was steep. This latter condition was achieved

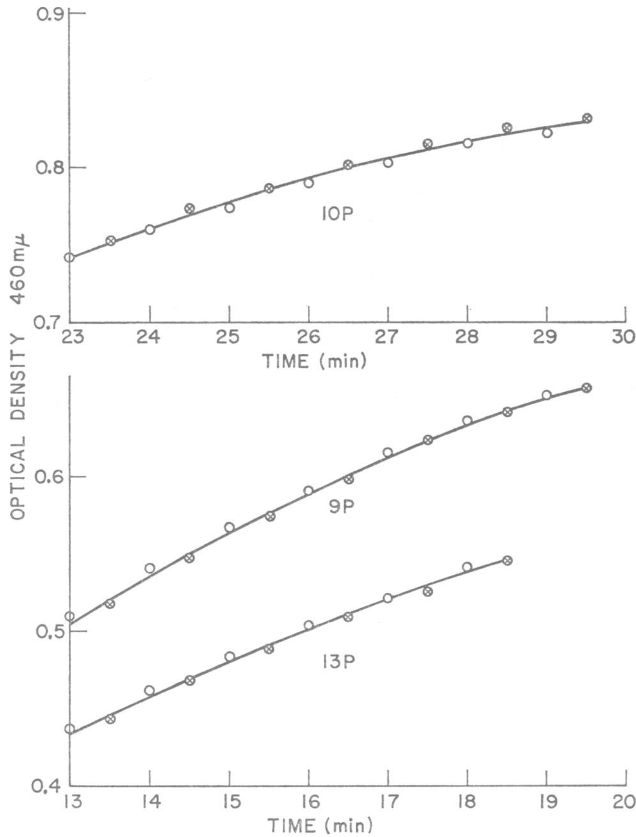

FIGURE 14 Pase reaction rate at 25°C. Effect of magnetic field of 170,000 gauss. ○, magnet; ⊗, control. 10P, 20 min exposure; 9P, 10 min exposure; 13P, simulated run, 10 min exposure. Enzyme concentration 7.5 × 10^{-6} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

by partial withdrawal of the pyrex reaction tube from the magnet center to the edge of the magnet bore.

The apparent discrepancy between the initial OD (13 min) for the 10 min simulated run and the 10 min magnet runs is readily understood in terms of the lability of Pase in dilute solution. The following Table I compares readings at 13 min for these runs and the circumstances under which the solutions were kept.

These data are consistent with an activation energy for the deactivation process of 2600 cal/mole.

All the data shown here are clearly satisfied by a single curve for each magnet and control run, and while there is a somewhat wider divergence shown in Fig. 15, it is still within $\pm 2\%$.

(*c*) *Tyrosinase.* All the results for Tase are gathered together in one place, Fig. 16, where the data for two simulated and two magnet runs for 3 min exposure

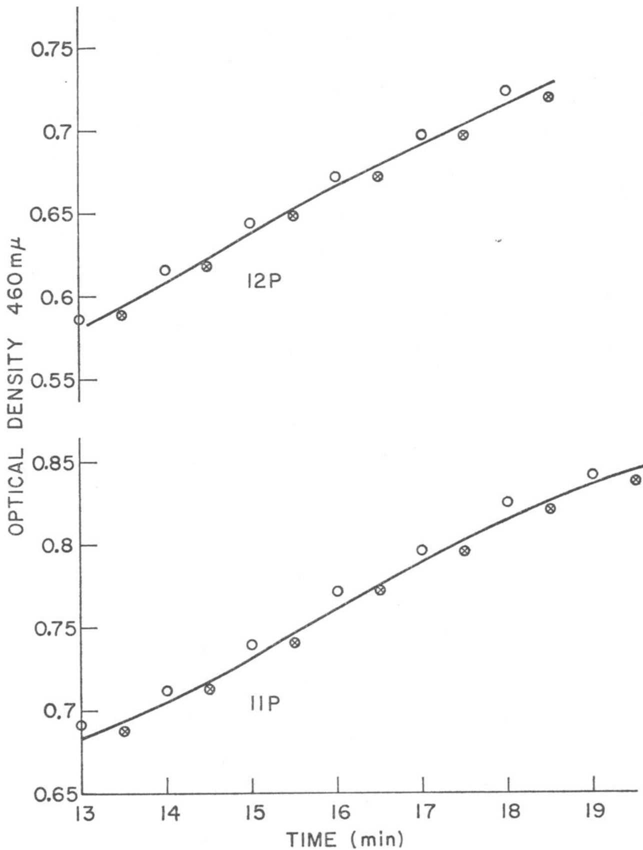

FIGURE 15 Pase reaction rate at 25°C. Effect of magnetic field. ○, magnet; ⊗, control. 12P, 10 min exposure to 85,000 gauss; 11P, 10 min exposure to large field gradient. Enzyme concentration 7.5×10^{-6} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

are normalized to the same OD value at 6 min. This normalized value is the mean of the actual run values and is 0.382 ± 0.027 where the quoted error is the mean of the deviations from the mean. The reason for this 7% deviation can be seen (Fig. 5) from the existence of an induction period which, under the conditions of our experiments, is poorly defined and not easily reproducible.

In Fig. 16, we have also included a 7 min simulated and a 7 min magnet run

which have been normalized to a common OD value at 10 min. This common value was taken as the mean of the 10 min values of the four normalized 3-min runs which gave 0.562 ± 0.008.

The two lower sets of points at the longest times are considered to be invalid runs and appear to have suffered from an inhibitory reaction. This is deduced from the abrupt nature of their conclusion and their markedly lower end points. In neglecting these in later considerations, it will be noted that both a simulated run and a magnet run are represented. Thus no bias is thereby introduced into any qualitative deductions.

TABLE I
LABILITY OF PEROXIDASE

Run	Initial OD	Remarks
8*P*	0.595	at 25°C for 18 min
9*P*	0.510	at 25°C for 31 min
11*P*	0.692	at 0°C for 66 min
12*P*	0.587	at 0°C for 90 min
13*P*	0.437	at 0°C for 113 min

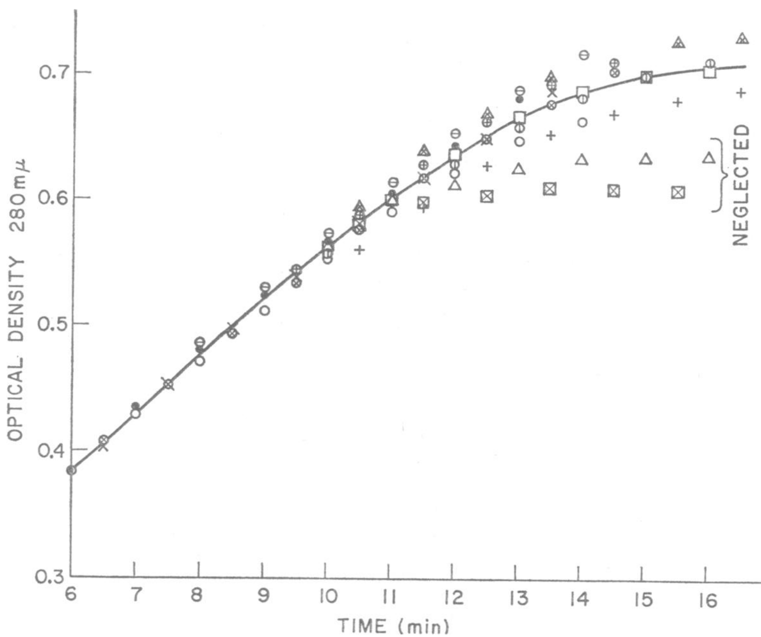

FIGURE 16 Tase reaction rates at 25°C. Effect of magnetic field of 170,000 gauss. Simulated runs, 3 min exposure: ○ and ⊗, magnet and control; ● and ×, magnet and control. Magnet runs, 3 min exposure: ⦶ and +, magnet and control; ⊖ and ⊕, magnet and control. Simulated run, 7 min exposure: △ and ⚠, magnet and control. Magnet run, 7 min exposure: □ and ⊠, magnet and control. Enzyme concentration 9.7 × 10^{-2} mg/ml before adding substrate. Reactions placed in magnet at 2 min.

When these *two* doubtful runs are ignored, the mean course of the remaining *ten* is given by the full line in Fig. 16, and it can be seen that this line satisfies the points to within ±3% in optical density.

(*d*) *Aldolase*. The results of the work done with Aase are shown in Figs.

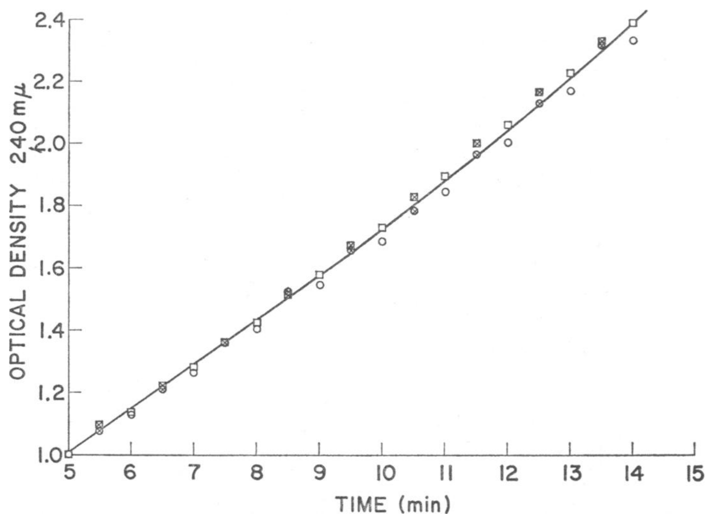

FIGURE 17 Aase reaction rate at 25°C. Effect of magnetic field of 170,000 gauss. Simulated run, 2 min exposure: ○ and ⊗, magnet and control. Magnet run, 2 min exposure: □ and ⊠, magnet and control. Enzyme concentration 6.05 × 10^{-3} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

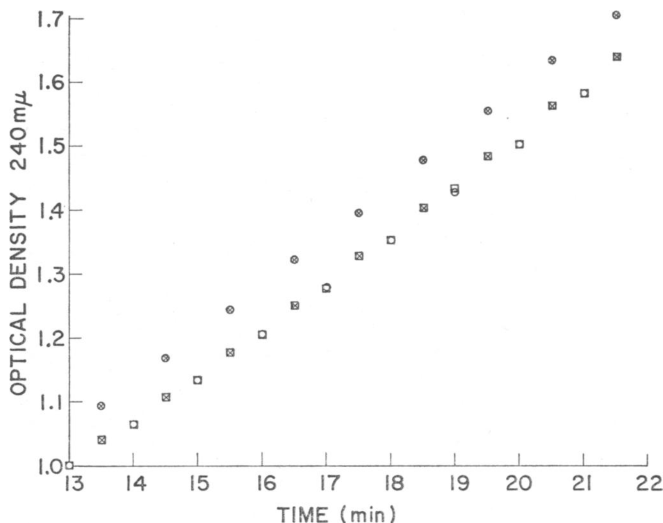

FIGURE 18 Aase reaction rate at 25°C. Simulated runs, 10 min exposure: ○ and ⊗, □ and ⊠, magnet and control. Enzyme concentration 6.05 × 10^{-3} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

17–19. A comparison of a 2 min simulated run with a 2 min magnet run (Fig. 17), normalized to an OD of 1.0 at 5 min, clearly shows data that can be satisfied by a single line. A comparison of two simulated 10-min runs, again normalized to an OD of 1.0 at 13 min, (Fig. 18) shows a control reaction displaced along the optical density axis by a distance of close to 0.06 in OD (i.e., 5%) from the line that satisfies the other three sets of points.

In Fig. 19, three 10-min magnet runs are displayed and while five sets of points normalized to an OD of 1.0 at 13 min, are very well satisfied by a single line, which is in close agreement with the line of Fig. 18, one set of points, a control reaction, deviates markedly from it. Although we cannot offer a ready explanation for this

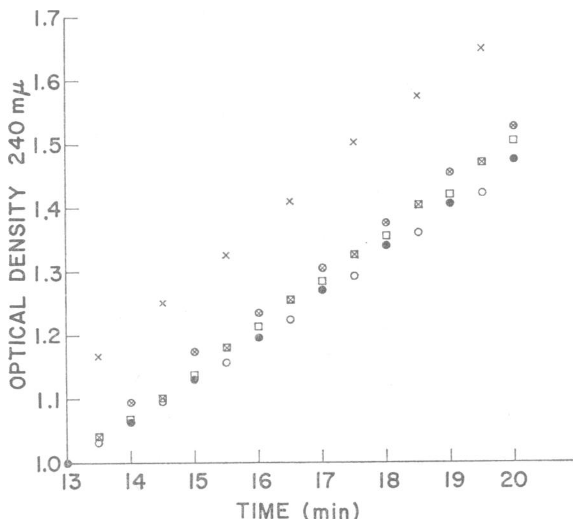

FIGURE 19 Aase reaction rate at 25°C. Effect of magnetic field of 170,000 gauss. Magnet runs, 10 min exposure: ○ and ⊗, ● and ×, □ and ⊠, magnet and control. Enzyme concentration 6.05 × 10^{-3} mg/ml in reaction mixture. Reactions placed in magnet at 2 min.

deviation, in light of the good conformity of two other control reactions, we cannot regard it as highly significant.

The justification for normalizing these data to a common starting point arises from the existence not only of an inductive period, but also of a nonenzymatic reaction rate for this reaction (Fig. 7). These two sources of error are likely to produce displacements of the course of reaction an ill-defined distance along the time scale.

DISCUSSION

With the possible exception of the aldolase reaction, it seems unequivocally clear from the data that, within the limits of error of this experimental technique (±3%),

there is no identifiable effect of magnetic fields on either the rate or course of the reactions of the enzyme systems examined. In the instance of aldolase, the case for an effect is so tenuous that it seems to us more appropriate to assign the deviation from a single line to an increased experimental error (10%) and again to conclude that, to this limit, there is no noticeable effect.

In the cases of RNase and Pase, the possibility of two opposing effects produced by the field is ruled out by the absence of any noticeable effects at fields of 100 and 85 kgauss respectively.

This conclusion must be extended to the case of an applied field gradient in the Pase reaction where we conceived of the possibility of a change in collision frequency between enzyme and substrate. This would arise if the field gradient affected the normal distribution of thermal energies of the molecules by superimposing a preferred direction of migration of dipolar species.

The discrepancy between the results of Cook and Smith (3), of Smith,[1] and of Wiley et al. (4), and indeed of Akoyunoglou (8), and the present authors might be accounted for in several ways. First, the earlier work used periods of hours for exposure times to the magnetic field whereas we have used only minutes. However, we believe that this must, at least, be partially compensated for by the incomparably higher fields used here. These fields are the highest ever used with enzyme systems, and while times as short as 2 or 3 min exposure were used, so also was a 20 min exposure in the case of Pase.

Second, it might be that the effect showed up in the enzymes used by the earlier workers, namely chymotrypsin, trypsin, and carboxydismutase, but not in ours. It must be said, however, that the experiments of these other authors differed somewhat from ours. They all exposed their enzymes to a "pretreatment" in the magnetic field before allowing them to react with their respective substrates, a reaction which takes place in the absence of an applied field. Only if it transpires that the enzyme molecule can be affected by the applied field but not the enzyme-substrate complex, would this nullify a comparison. Nevertheless, we plan to carry out such a series of pretreatment experiments.

Third, the results of the earlier workers might be a consequence of temperature differences between samples exposed to magnetic fields and samples (controls) not exposed. We do not know, nor do we mean to imply, that this is the case, but it is instructive to consider the so-called activation energy (μ) of the heat deactivation (denaturation) process of the enzyme. Putnam (9) quotes values of 40–100 kcal/mole for μ as being typical and values as high as 200 kcal/mole as possible. This means that a 24% change in reaction rate could be accounted for by temperature differences in the range 0.4° to 1.0°C.

It is our belief that, if a biomagnetic effect exists for enzymes, it will either be very small and observable only by exposures to fields of strength comparable to those used here, and for periods of hours, or, the onset of a critical change in enzyme kinetics will occur only at much higher fields.

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