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Evidence for a Non-Linear Relationship Between β-Galactosidase Activity and Enzyme Concentration Due to the Loss of an Activator During Ammonium Sulphate Precipitation

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It has been observed that the activity of β-galactosidase precipitated with ammonium sulphate drops non-linearly in dilutions of Tris (10mM, pH 8.0). We sought to provide evidence for this trend, henceforth referred to as the "dilution effect," as well as possible explanations for this phenomenon. We theorized that this dilution effect was due to the possible loss of activators during the precipitation process in the purification protocol employed in a previous experiment. We diluted the ammonium sulphate precipitated enzyme with Tris to reproduce the dilution effect. Next, we compared the rates at different substrate concentration using Tris or an alternate diluent consisting of filtered cell components less than 30,000 MW. Using the rates obtained from these assays, a Lineweaver-Burk transformation of the Michaelis-Menten equation was done to determine V_{Max} and K_M. In the ammonium sulphate precipitated enzyme, we discovered that the contents of the filtrate under 30,000 MW increased affinity but caused no significant change in V_{Max}. We believe that this is evidence of an activator that is less than 30,000 Daltons present in the IPTG induced cells of Escherichia coli.

β-galactosidase is a tetramer of molecular weight 540 kDa, made up of four identical subunits (9). We observed a disproportionate loss in enzyme activity during the purification process when the enzyme was diluted, which we have termed the "dilution effect." The dilution effect was observed with ammonium sulphate precipitated enzyme (6).

It has also been observed by Contaxis *et. al* that as β-galactosidase is purified through an ion-exchange column (e.g. DEAE), there is a "marked increase in 280/260 ratio [but] a loss of total units of activity greater than expected" (2). The unexpected decrease in activity may be due to the loss of a molecule present in the crude extract, but removed through ion-exchange chromatography. The molecule was believed to decrease the dissociation constant of the (active) tetrameric form to the inactive dimeric form. Mg^{+2} ions are well known to be co-factors for β-galactosidase (8).

In this study we examined the reproducibility of the "dilution effect", then we tested potential explanations by showing a difference between diluting both the β-galactosidase present in the filtered broken cell and an ammonium sulphate (AS) precipitated form of the β-galactosidase with either Tris or a filtered form of the crude extract (MP3) as diluent. MP3 more closely resembles the environment of the cell and contains any low molecular weight molecules (e.g. cofactors, ligands, activating/inhibiting molecules) present in the cell which could affect the observed activity of the enzyme.

METHODS AND MATERIALS

Bacterial Strain. Overnight cultures of Escherichia coli C29 were grown in modified Luria Broth containing 0.4% glycerol (in place of glucose) in a 37˚C shaking water bath. The culture was then induced with IPTG (100 mg/ml) to give a final concentration of 280 µg/ml (6) in Luria broth and allowed to grow for 4- 5 more hours.

Preparation of the Crude Cell Extract/Filtered Broken Cell. Cells were lysed using the alumina grinding method, as described in the Microbiology 421 Lab Manual (6). Separation of the bacterial cells from the broth involved centrifugation at 7000 x g for 10 minutes. After the dry-ice acetone bath and alumina grinding, centrifugation at 7500 x g was used to pellet unbroken cells. Deoxyribonucleases (300 µg/ml) and ribonucleases (300 µg/ml) were both added to reduce nucleic acid contamination. Tris buffer (pH 8.0, 10 mM) was used for the diluent and the solvent.

Purification of β-galactosidase. The enzyme was partially purified by ammonium sulphate precipitation and DEAE-chromatography according to the Microbiology 421 Lab Manual (6). Initially, β-galactosidase was purified further using Sephadex and DEAE column chromatography. The Sephadex G-150 column has a nominal fractionation range of 5 to 3000 kDa. The pellet suspension from the ammonium sulphate precipitation was run through the column. The approximate protein concentrations, as well as the A_{260} and A_{280} readings or each fraction were measured. The

DEAE mini column chromatography protocol (Dr. W. Ramey, personal communication) was a modified version of the DEAE Column. We used the mini-spin columns and rinsed them with varying concentrations of TN buffer in a microfuge at 5000 x g for 2 minutes. However, this purification produced low yields of the enzyme and was not used for further analysis.

B-galactosidase assays. A modified β-galactosidase protocol (7) was used for measuring rate of enzyme activity, as the volume mentioned in Microbiology 421 Lab Manual (6) was too low to measure prior to the addition of Na_2CO_3. However, we used Tris (pH 8.0, 10 mM) instead of potassium phosphate buffer, in order to be more consistent with the protocol in the Microbiology 421 Lab Manual (6), where the dilution affect was observed. Various enzyme concentrations were initially assayed with this assay protocol (undiluted as well as dilutions of AS-precipitated enzyme at 1/2, and 1/5) as specified in the protocol. This was done to determine a linear relationship between observed activity (units/ml) versus enzyme quantity (dilutions relative to the undiluted enzyme). To determine K_M and V_{Max}, the substrate concentration was varied to construct the Lineweaver-Burk plot. We started with the addition of water (1.0 ml), Tris (1.5 mL), and diluted/undiluted ONPG substrate (1.0 mL) to the cuvette. The last step was adding the enzyme (0.1 ml), and both the time and absorbance were recorded from the instant the enzyme was added to the cuvette. For dilutions, we varied ONPG concentration using the 30,000 fraction or Tris as diluent (henceforth referred to as the MP3 or TRIS treatments). Dilutions of 1/2, 1/4, 1/8, 1/10, and 1/20 were made of ONPG. Absorbance was measured using a Spectronic 20 spectrophotometer, and readings were recorded on a Spec X (Vernier Inc., Seattle) computer program running on a 386 based PC running MS-DOS. We determined reaction rates by setting the computer to measure $A_{420\ nm}$ every 0.5 seconds for an interval of 30-60 seconds. As mentioned, timing began once the enzyme was added to the assay tube (cuvette). The rates were obtained by taking the first 10 readings after placing the cuvette into the Spectophotometer (when the substrate concentration would be highest, thus the observed rate would be highest, as well. With regards to the assay, the pH was assumed to be the same between all treatments and conditions, although we did not explicitly test for it. Also, we assumed the temperature to be constant, which may or may not have affected our particular assays due to fluctuations in room temperature (people opening windows, sunny days, etc.). All subsequent rate data would thus be affected by these factors.

RESULTS

Evidence for the Non-linear Relationship of Enzyme Activity and AS Precipitated Enzyme. Figure 1 shows a fairly linear relationship between enzyme activity (measured as change in absorbance at 420 nm over time) and enzyme quantity (measured in relative to the undiluted AS precipitated enzyme) through the undiluted, 1/5, and ½ dilutions. However, at an "infinite" dilution (i.e. no enzyme present), activity must be zero. Therefore, it appears based on this linear trend that the observed activities are greater than expected in these dilutions.

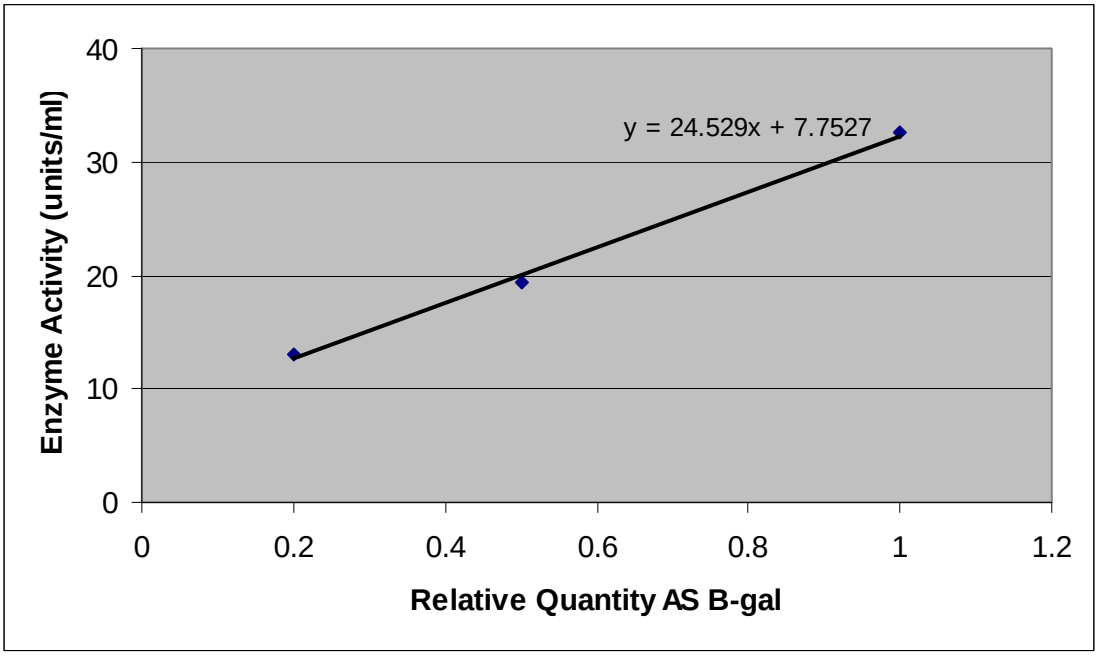

FIG 1. The observed activity over various concentrations of ammonium sulphate precipitated B-galactosidase.

Testing the MP3 fraction for β-galactosidase activity. No activity was observed when the MP3 fraction was tested using the β-galactosidase activity assay. Therefore, we can safely assume that any changes in the rate between the TRIS and MP3 treatments are not due to additional enzyme present in the MP3 fraction (unpublished data).

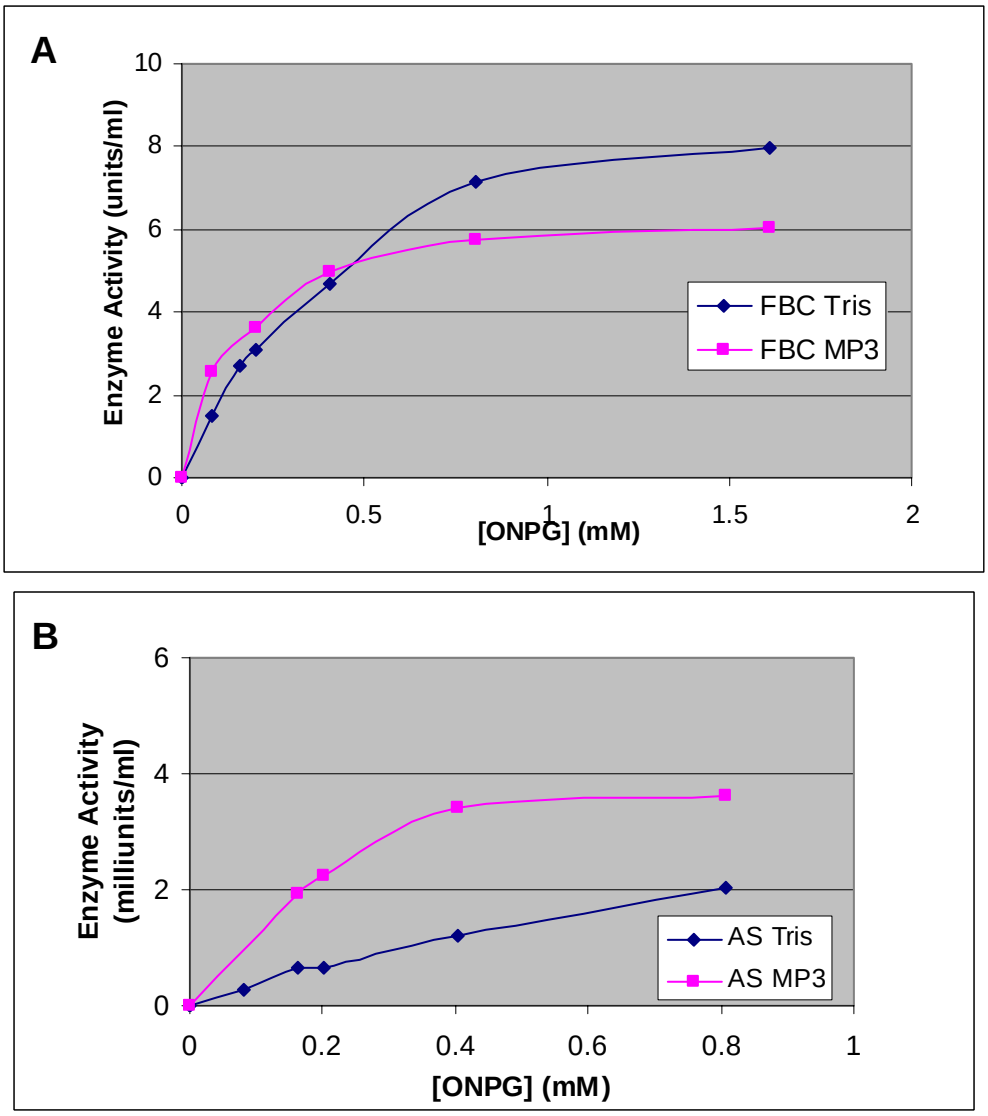

FIG 2. The measured activity over time at various ONPG concentrations with (A) enzyme from the Filtered Broken Cell and (B) enzyme from the ammonium sulphate precipitation.

Enzyme Activity in the Filtered Broken Cell Fraction (FBC). With the filtered broken cell extract, there was only a slight difference between the TRIS and MP3 treatments in the low concentrations of ONPG substrate. Rates in the MP3 treated sample were slightly higher in the 1/20 and 1/10 dilutions of ONPG. With a high substrate concentration however (1/2 dilution of ONPG), the observed rate in the assay diluted in MP3 was much lower than the observed rate in TRIS with the same dilution level (Fig 2A). It appears that in the FBC treatment, MP3 resulted in a slight increase in rate and only at low concentrations of substrate and high concentrations of MP3.

The graphically determined V_{Max} for TRIS was greater than the V_{Max} for MP3. However, the MP3 treatment resulted in a lower K_M than the TRIS treatment. The difference between the two K_M values was much greater than the differences between V_{Max} (Table 1).

The enzyme activity in ammonium sulphate precipitated enzyme fraction (AS), on the other hand, was significantly different between TRIS and MP3 across all dilution levels of substrate (Fig 2B). The MP3 treatment assays had greater rates at all concentrations of ONPG, and a greater change in rates between all changes in substrate concentration. In other words, the rate of change of velocity per change in substrate concentration was consistently greater in MP3 than TRIS. For MP3, changes in rate were smallest between the 1/2 dilution and 1/4

dilution. The greatest change occurs between 1/20 and 1/10 dilutions. For TRIS, the change in velocity over change in relative substrate concentration appeared to increase linearly regardless of the current dilution levels.

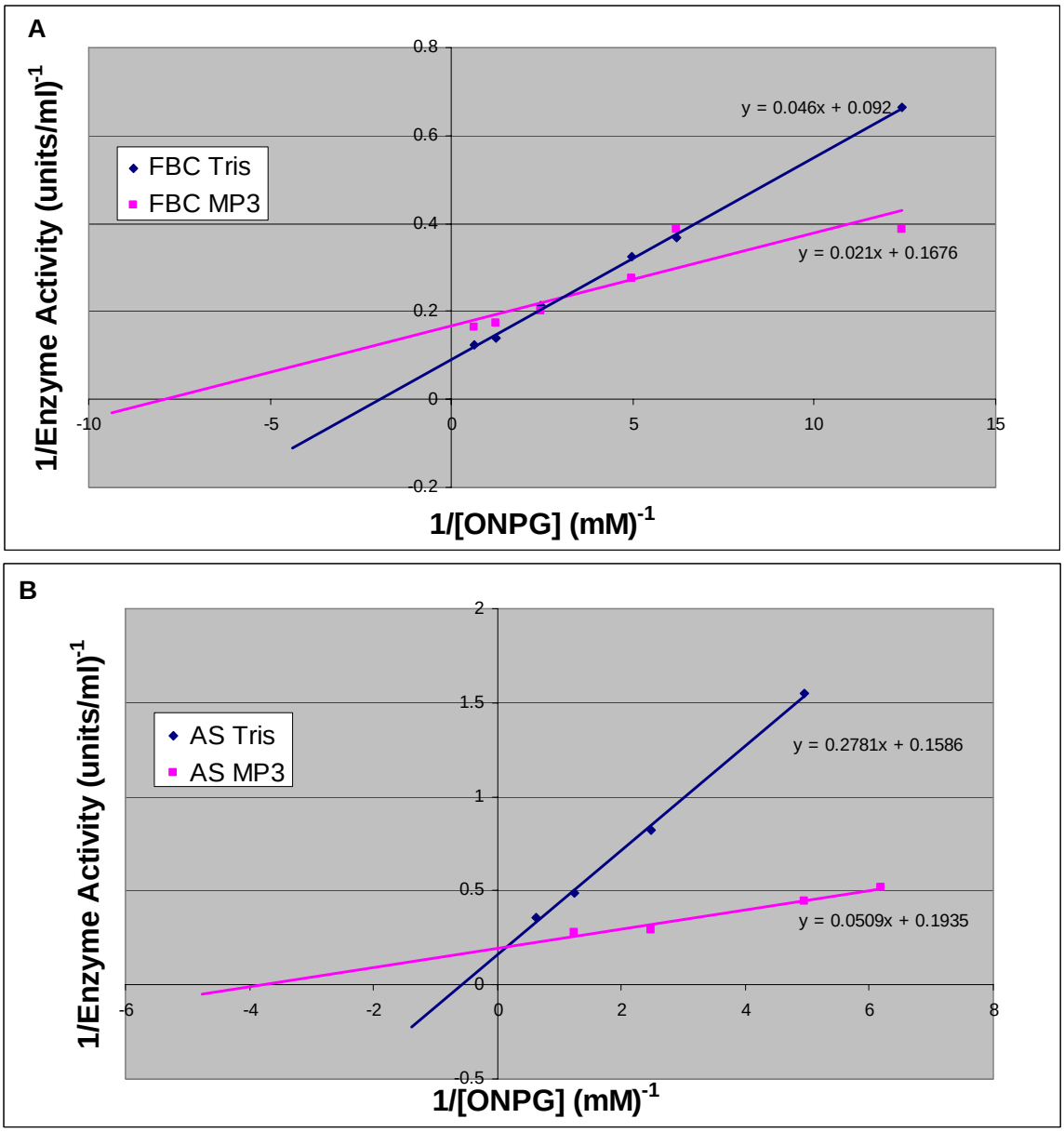

FIG 3. Lineweaver-Burk plots of 1/enzyme activity and 1/[S] to determine the theoretical maximum rate (V_{Max}) and K_M values for (A) Filtered broken cells and (B) ammonium sulphate samples. Ammonium sulphate precipitated enzyme results in a decrease in both affinity and maximum rate, irrespective of the diluent used.

Table 1. Summary of the results from Lineweaver-Burk plots

Sample	K_m (mM)	V_{Max} (units/mL)
FBC TRIS	0.50	10.0
FBC MP3	0.13	6.0
AS TRIS	1.75	6.3
AS MP3	0.26	5.1

Compared to TRIS, the MP3 treatment resulted in a higher $1/K_M$, and a slightly higher $1/V_{Max}$ with the AS precipitated enzyme. This translated to a lower K_M and a slightly lower V_{Max} in MP3 treatment vs. the TRIS treatment. K_M is 6.7 times greater in TRIS than MP3, while V_{Max} is only 1.2 timestimes greater in TRIS (Table 1). Therefore, it appears that the differences in substrate affinity were far more significant than the differences in V_{Max}.

General Trends Observed between FBC and AS enzymes. In the assays with the AS precipitated enzyme, a significant decrease of the rates was observed in both diluents at all dilution levels (see Figs 2 and 3A), and consequently a decrease in V_{Max} and an increase in K_M. In both cases, ammonium sulphate lowered the maximum rate of reaction and the affinity for substrate when compared to the FBC treatment. It is important to note that the amount of enzyme activity was not controlled for the FBC and AS enzymes, and this could very well explain the differences observed between these two treatments. The denaturing property of ammonium sulphate could also lower effective enzyme concentration (Dr. W. Ramey, personal communication), and it is well known that salt also affects enzyme activity. Without controlling for these confounding variables, we cannot determine the exact reason for the decrease in V_{Max}. These explanations are obviously not mutually exclusive, and it could very well be a combination of factors that could account for these differences (in addition to experimental error).

DISCUSSION

It is obvious from Fig. 1 that a linear relationship exists within a certain range. If this trend continues, there would still be activity in the absence of any enzyme. Therefore, the amount of enzyme activity observed is greater than expected when diluted. This is contrary to the "dilution effect" observed by other students in the lab.

The differences observed between activity over various enzyme concentrations could be due to the quality of the ammonium sulphate precipitation. Perhaps some students eliminated the activator during the ammonium sulphate precipitations. The absence of the activator could explain the disproportionate loss of enzyme activity observed during dilutions. An exploration of the differences of activity observed between TRIS and MP3 dilutions provides a possible explanation.

TRIS is an ideal buffer for β-galactosidase assays as it acts as a nucleophile to facilitate the release of galactose from the ONPG-enzyme complex (8). It attaches to ONP-Galactose-Enzyme Complex, breaking the bond between galactose and ONP more efficiently by nucleophilic interaction with the electrophilic galactose, increasing the rate of product formation, but leaving substrate affinity unaffected (Dr. Ramey – personal communication). This provides a plausible explanation for the greater V_{Max} as well as the lower K_M observed in the FBC-TRIS treatment when compared to FBC-MP3.

When using FBC enzyme and MP3 as a diluent, apparent K_M was reduced, as well as V_{Max} when compared to the respective values in FBC-TRIS. In theory, an activator would increase the affinity of B-galactosidase for its substrate, decreasing in K_M, and the theoretical maximum rate would not be altered. However, it appears that with the FBC enzyme, V_{Max} is significantly affected by the use of MP3 as a diluent relative to TRIS. FBC-TRIS V_{Max} is 1.6 times greater than FBC-MP3 V_{Max} (Table 1). Nevertheless, the decrease in K_M was much more significant than the decrease in V_{Max}. V_{Max} could also be affected by pipetting errors (changing the concentration of enzyme), but is insufficient to explain the differences observed here. However, it is very plausible that a significant amount of the difference in V_{Max} values could be explained by the absence of the nucleophilic properties of TRIS molecules. Therefore, it is not unreasonable to assume that the primary difference between the FBC-MP3 and FBC-TRIS treatments was substrate affinity. Thus, it appears that there is an activator present in MP3 that is increasing the rate of formation of the ES complex (increasing the association constant of ES).

Ammonium sulphate is known to inhibit the nucleophilicity of TRIS (Dr. Ramey – personal communication). In this case, we would expect no significant difference between the V_{Max} of AS-TRIS and AS-MP3. This is exactly what we observed when comparing the AS-MP3 and AS-TRIS treatments. The slight differences of V_{Max} could easily explained by the slight change in enzyme concentrations due to pipetting and other sources of error.

A significant difference in the K_M is evident between the AS-TRIS and AS-MP3 treatments. Furthermore, this difference was much larger than the difference in K_M between FBC-TRIS and FBC-MP3. This can be explained by the composition of the FBC obtained enzyme. FBC contains all molecules present in the cell, including the material less than 30,000 MW found in MP3. This would account for the smaller difference in K_M's between FBC-TRIS and FBC-MP3 (the FBC-MP3 would still contain more activators overall as it contains the activator present in FBC in addition to the activators present in MP3 diluent). However, if these activators did not precipitate through ammonium sulphate, AS-TRIS would not contain a significant amount of these activators. This provides further evidence that molecules present in the MP3 fraction are the cause of increased affinity of β-galactosidase for substrate, resulting in a lower K_M.

K_M is reduced, as evident in Figures 2a and b. This could be due to the possible presence of an activator in the MP3 fraction. In theory, such an activator would increase the affinity of β-galactosidase for its substrate, accounting for the decrease in K_M. In other words, there is a decrease in binding time (i.e. the time it takes E and S to find each other in solution and bind is decreased). However, the theoretical maximum rate would not be altered. As expected, we saw a decrease in K_M in the AS-MP3 fraction relative to the AS-TRIS fraction, and no significant difference in V_{Max} between them. Thus, the experimental results agree quite well with the results predicted by theory. Thus the activator is probably increasing the rate of formation of the ES complex.

In all cases, there is no evidence of allosteric interaction (no sigmodial curve observed during increase). In fact, all our assays showed classic M-M kinetics (constructed through various substrate concentrations). It is important to stress that these results only show that the presence of an activator is plausible, and due to the complex nature of enzyme regulation and kinetics, many other possibilities and explanations must be tested.

Our results show strong support for greater than expected activity when ammonium sulphate precipitated β-galactosidase is diluted, contrary to the observations made earlier. We also find evidence of differences in activity between the uses of different diluents for ONPG (MP3 vs. TRIS). In the AS-MP3 treatment compared to the AS-TRIS treatment, we observed a drop in K_M and a much less significant drop in V_{Max}. We believe that this is evidence for the presence of an activator in the 30,000 MW fraction of the filtered broken cell. We propose that the activator serves to increase the affinity of β-galactosidase to its substrate, resulting in a faster rate at low concentrations of substrate or enzyme, but would not affect the theoretical maximum rate. The presence of this activator and its dilution will undoubtedly impinge on the observed rates of activity in different dilutions of enzyme, possibly accounting for the dilution effect. Due to the size of the filter used, the material present in the MP3 diluent could very well contain the adenosine-derivative ligand discovered by Contaxis et. al (2), and can not be ruled out as a possible reason for the observed increase in activity.

FUTURE EXPERIMENTS

Due to the constraints of time and resources, further study must be done to characterize the dilution effect and the proposed activator. Certainly, it is important that we repeat our experiment, but with some minor modifications. First of all, the enzyme concentration should be determined, possibly by using a Bradford assay. The enzyme concentration is important for determining the K_{cat}, and for doing comparable analysis between the ammonium sulphate and the filtered broken cell. It may also be important to use other E. coli strains that may be more reliable, or produce higher yields of β-Galactosidase. During the assays, it is also important to control for the affects of Tris to accurately determine the affect of the diluent. Varying the concentration of the proposed activator (MP3), while keeping all other variables constant, will also give further insight into the effects of MP3. Finally, it may be best to modify the protocol by using a different method to break open the cells, such as sonication. This would have a less adverse affect on the cells and perhaps yield a greater amount of β-Galactosidase.

Some further experiment based upon our analysis would involve characterizing and possibly isolating the putative activator. To characterize the activator, the diluent could be heated then added to the assay, to identify whether it is a protein. If the activation effect persists when it is used as a diluent, then the activator was not disturbed by heat, and is probably not a protein. The diluent could also be processed through an ion-exchange column before being used as a diluent in the assay. If the activation effect is lost, then it may be it as an ionic cofactor. Another possibility for characterization and purification include the use of a radiolabelled nucleotide, such as ^{14}C, incorporated into the growth media could also be used, in conjunction with thin layer chromatography,

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