The Induction of Tyrosine Aminotransferase from Rat Hepatocytes

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Abstract

It was found that several different hormones, separately and together, induced the enzyme tyrosine aminotransferase, which resides in cells of the liver. A modified Diamondstone assay was used to determine the enzyme’s activity and the Lowry assay was used to determine protein content. Insulin alone showed the smallest amount of induction, peaking at 278% of the zero hour control. Dibutyryl-cAMP, an analogue of cAMP, and dexamethasone showed a high level of induction, peaking at 328% and 335%, respectively. Dibutyryl-cAMP, dexamethasone and insulin together showed the strongest induction, hence the hormones’ inductive effect were additive. The highest level of induction (612%) was the 7½-hour enzyme sample that included all the hormones. The change in the control’s activity over time was unremarkable, showing only minute activity change over time. All the conditions had a low induction potential at the start (1.5 hours after addition of hormone), and their induction activity dipped overnight. The exception to this is dibutyryl-cAMP which showed its strongest induction after the overnight period. It was also found that all the components, including diethyldithiocarbamic acid and pyridoxal-phosphate were required in all assays. Without them, only a fraction of the activity of tyrosine aminotransferase was observed compared to when they were included.

Introduction

There is a wealth of research over more than half a decade using rat hepatocyte cells. In this project, I will investigate an enzyme that appears in eukaryotic liver cells in the mitochondria and cytosol [1], known as tyrosine aminotransferase (TAT; EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase) [4]. It is a cationic enzyme with a molecular weight of approx. 90k, determined by Litwick and Miller by amino acid composition and molecular sieve chromatography [1]. It is also found in Homo sapiens and other mammals.

This enzyme is also called tyrosine transaminase, named for its transamination activity on tyrosine [2, 12]. Transamination is described as the “transfer of an amino group from an amino acid to a keto acid” [3], and hepatic tyrosine aminotransferase transfers a nitrogenous group from...
L-tyrosine to 2-oxoglutarate (α-ketoglutarate) to form 4-hydroxyphenylpyruvate and L-glutamate [2, 3]. This is a catabolic and reversible reaction; the first step in the tyrosine pathway. Pyridoxal-phosphate is a co-factor in this reaction, as many transferases require a co-factor to function fully [3]. These components will be explained in detail later in this project.

This study will focus on measuring the activity of tyrosine aminotransferase of non-induced and hormone induced hepatocyte monolayers. The assay used in this project is to determine the enzymatic activity of TAT using L-tyrosine and α-ketoglutarate as substrates. The assay is known as the Diamondstone assay [4], with some minor noteworthy changes from the original that will be described in the discussion section. Diamondstone explained that a low concentration of TAT is needed to measure its activity, and that the Brigg’s colorimetric assay [cited: 5] was neither sensitive nor accurate enough at these concentrations. Diamondstone found that converting p-Hydroxyphenylpyruvate to p-Hydroxybenzaldehyde gave “a rapid, convenient, and sensitive fixed-time assay” to which a 15- to 20- fold increase in sensitivity was ascertained [4]. This assay included use of diethyldithiocarbamic acid (DDC), pyridoxal-phosphate (PLP), α-ketoglutarate and 10M Sodium hydroxide (NaOH). Diamondstone initially included trichloroacetic acid (TCA), which was used in an earlier experiment by Lumeng (1978), to terminate the reaction [6]. However, Diamondstone found that coupled with the other components, NaOH and DDC, that the blanks were very high and therefore added inaccuracy to the experiment. For this reason, the use of TCA will be omitted from this project, as Diamondstone had done [4]. 10M Sodium hydroxide quenches the activity of tyrosine aminotransferase and prevents the reverse reaction from taking place, because the products of this reaction are not removed from the mixture. Thirty minutes after the addition of 10M NaOH should be adequate to convert p-Hydroxyphenylpyruvate into a readable product at 331nm.

The hormones that will be used in this project are insulin, dexamethasone and dibutyryl cyclic adenosine 3′,5′-monophosphate (dibutyryl-cAMP). Their ability to induce TAT will be determined using the modified Diamondstone assay. Evans PJ (1981) stated that tyrosine aminotransferase was an unusual enzyme due to its ability to be “induced many-fold over its basal activity” and that the effects can occur in perfused liver [29].

An enzyme called 4-hydroxyphenylpyruvate dioxygenase is also involved in the tyrosine pathway. It converts 4-hydroxyphenylpyruvate into homogentisate using oxygen (oxidoreductase). The enzyme’s cofactor is iron [7]. This enzyme presents a problem with this assay since it converts the product of TAT into an unreadable product. Therefore, diethyldithiocarbamic acid (sodium diethyldithiocarbamate) is used in the assay to inhibit 4-
hydroxyphenylpyruvate dioxygenase. This is supported by the work of Lin et al., who showed that the addition of DDC eliminated the activity of the oxidoreductase, and therefore further degradation of tyrosine does not occur. [8]

The cofactor pyridoxal-phosphate (PLP; vitamin B6; C_{8}H_{10}NO_{6}P) is naturally found in the cytosol and its binding to tyrosine aminotransferase is essential to the enzyme’s activity [9]. Therefore, it will be included in this assay (similarly in the assay performed by Diamondstone) [4]. Canellakis & Cohen described the activity of PLP in their experiment in 1956, they showed that increasing the level of PLP (in µg) increased the μM of p-hydroxyphenylpyruvic acid – a very small amount of pyridoxal-phosphate raised the activity to 2% from maximum [10]. Hayashi et al. showed that very little of pyridoxal-phosphate is needed to saturate the PLP binding-site of TAT, 1 mole of PLP bound per 94,000g of enzyme. They theorized, based on this information, that tyrosine aminotransferase has different subunits and that PLP binds to just 1 subunit [11]. This theory is beyond the scope of this project, and will not be investigated.

2-Oxoglutarate is a substrate in this reaction hence it should be included. Tyrosine aminotransferase transfers an amine group from a molecule of L-tyrosine onto a molecule of 2-oxoglutarate at 37 °C. Preparation of this substrate requires pH adjustment by 1.5M NaOH alkaline solution, this method is supported by the work of Cammarata and Cohen (1950) who researched transamination reactions [12]. The preparation of this substance is described in detail later. Schepartz (1951) described α-ketoglutarate as “a limiting factor in the catabolism of tyrosine because it was acting as an acceptor of amino groups” [13].

Insulin is a protein that has hormonal activity in mammalian systems and it comprises of 51 amino acids linked by disulphide bonds [3]. One of its functions is the regulation of blood-glucose levels as an opposite of glucagon; it reduces the level of glucose by converting it to glycogen. Insulin’s activity on tyrosine aminotransferase is well documented in scientific journals. One such example is from Pittner et al. (1985), who experimented with monolayer rat hepatocytes using a modified Leibovitz L-15 medium. They introduced insulin to measure any increase in the activity of tyrosine aminotransferase, and whether or not it affects the activity in conjunction with dexamethasone and dibutyryl-cAMP [14]. One area of this project is to determine whether I can achieve induction of TAT with the method and insulin concentrations that I used. I would also explore how insulin behaves when included with the other hormones.

Dexamethasone is synthetic glucocorticoid that is used mainly as an anti-inflammatory and allergic agent; it also appears to have application in certain cases of cancer treatment [15, 16]. It is similar to a naturally synthesized hormone from the adrenal gland [16]. The drug’s
activity on tyrosine aminotransferase has been broadly researched and theorized in the literature; hence following accounts are just a minor part of the reports involving this subject area. Nitsch et al. (1993) found that dexamethasone has inducible activity on the TAT gene, exclusively in hepatocyte cells [17]. Aung & Ebner (1967) researched the initiation ability of several chemicals, including dexamethasone, on TAT [18]. Thompson et al. studied dexamethasone induction in hepatoma tissue culture cell lines in 1966 [19]. In this project, dexamethasone will be introduced on its own, with dibutyryl-cAMP and with both insulin and dibutyryl-cAMP.

Cyclic AMP or cyclic adenosine monophosphate is a secondary messenger and it is an important component in many biological systems as a regulator of metabolic processes [3, 20]. It is produced from ATP by adenylyl cyclase. The molecule can activate genes that consist of elements known as CRE [3]. The analogue of cyclic AMP, dibutyryl-cAMP, will be used in this experiment because it is able to penetrate through plasma membranes (which cAMP can not do). Since this experiment will be using hepatocyte monolayers, the cyclic AMP must be able to permeate the cells to gain any affect. Glucagon (a pancreatic hormone) stimulates adenylyl cyclase activity in mammals [21] hence it stimulates the production of cAMP. Dibutyryl-cAMP (db-cAMP) can therefore be described as a secondary messenger of glucagon, the important hormone that regulates blood-glucose. It has the opposite effect of insulin in an organism, so db-cAMP may have the opposite effect on TAT in this experiment and they may have conflicting action on each other. Wicks in 1968 originally researched the consequences of db-cAMP on tyrosine aminotransferase, published in Science journal [22]. Donavan and Oliver (1972) stated that cyclic AMP is involved in the mechanism to release tyrosine aminotransferase from polysome organelles [23]. Onoagbe et al. (1994) experimented using glucagon and cAMP analogues in chick embryos, to determine their regulatory effects on TAT [24].

**Materials and Methods**

The solutions made for the assay to determine tyrosine aminotransferase activity – there were a number of solutions made up. Each will be discussed in turn, including their storage instructions and when available, their original manufacturer.

**0.2M KPO4 (Potassium phosphate buffer) pH7.6:** The preparation requires 2 separate solutions - K2HPO4 and KH2PO4 solution; make-up instructions are included below. Both powders should be stored in a cool dry place. This method makes approximately 1L of buffer, and is a revised method. Place a 1L beaker and a flea onto a magnetic stirrer (with no added heat) and set the stir speed to 5-8. Pour in approximately 700mL of the K2HPO4 solution & activate
the magnetic stirrer. Pour the KH$_2$PO$_4$ solution in slowly, measuring the pH value of the gauge. Once the pH value has reduced to 7.6 then halt addition of the KH$_2$PO$_4$ solution. Store the 0.2M Potassium phosphate buffer pH7.6 at 4 degrees C, in a suitable container. Store unused K$_2$HPO$_4$ solution and KH$_2$PO$_4$ solution in the fridge too.

0.2M K$_2$HPO$_4$ solution: The molecular weight of K$_2$HPO$_4$ is $2(39.0983) + 1.00794 + 30.973761 + 4(15.9994) = 174.1759$. Therefore, to make up 1 litre of 0.2M K$_2$HPO$_4$ solution you measure out 34.8352g of K$_2$HPO$_4$ & place into a 1L beaker (add a flea). Add 800-900mL of distilled water and place on a magnetic stirrer. Use it to stir the solution until the K$_2$HPO$_4$ has dissolved. Pour the resulting solution into a 1L volumetric flask, wash the beaker with a small amount of water to obtain any remaining powder and pour it into the flask. Add extra water to the 1L marker and store in an appropriate plastic container.

0.2M KH$_2$PO$_4$ solution: The molecular weight of KH$_2$PO$_4$ is $39.0983 + 2(1.00794) + 30.973761 + 4(15.9994) = 136.0855$. Therefore you make up 500mL of 0.2M KH$_2$PO$_4$ solution by measuring out 13.6086g of solid, place into a 500mL beaker (and add a flea). Add 400-450mL of distilled water and place on a magnetic stirrer. Use it to stir the solution until the KH$_2$PO$_4$ has dissolved. Pour the resulting solution into a 500mL volumetric flask, wash the beaker with a small amount of water and pour it into the flask. Make it up to 500mL by adding extra water and store in an appropriately sized container.

Tyrosine-buffer solution: Tyrosine is very difficult to get into solution and the solution for the assay has tyrosine in excess. A solution of tyrosine requires constant agitation and approx. 35 degrees C of heat. This is the revised method and includes instructions for both 250mL and 500mL of total solution. To make 250mL of solution, measure out 0.27574g of tyrosine powder into a 500mL conical flask; to make 500mL, measure out 0.55148g of tyrosine. Fill approximately 220mL of 0.2M KPO$_4$ pH7.6 buffer into the flask and place on the hot-plate magnetic stirrer, place a flea into the beaker; for 500mL of solution, pour in 400-450mL of buffer instead. Cover the 500mL flask with a 250mL beaker and switch on the magnetic stirrer so it is on approximately 40 c and set it to spin at 5-6 speed, wait for 2-3 hours until the tyrosine goes into solution (or almost in solution). Enter the flask’s contents into a 250mL volumetric flask using a glass funnel, or a 500mL volumetric flask if you wish to make 500mL. Fill to the meniscus with 0.2M KPO$_4$ pH7.6. Pour the resulting contents into a 500mL conical flask and place on the hot-plate stirrer at a reduced temperature of 35 degrees C (but the same spin speed). Cover with the 250mL beaker so that none of the solution is evaporated; maintain the heat and stirring of the solution throughout the experimental procedure.
0.005M Pyridoxal-phosphate solution – This solution is light sensitive so it needs to stay in dark conditions. The solution is also stable at 4°C so it needs to be stored in the fridge, the solid medium needs to be kept in the freezer. Measure out 0.0309g of the solid into a weigh boat and transfer to a 25mL volumetric flask. Make up 25mL of solution by adding 0.2M Potassium phosphate buffer pH 7.6 into the flask and seal it. Shake so that all the solid material is dissolved. Transfer into a container that is covered with foil.

0.15M Diethyldithiocarbamic acid, sodium salt (DDC) – This solution breaks down after time so it needs to be made fresh daily. This compound is harmful if ingested in substantial quantities and it stains the skin yellow in direct contact. It is also harmful to the eyes and should be washed out with copious amounts of water over a 10-minute period, if exposed. Wear safety spectacles plastic gloves throughout. After disposal, the container needs to be rinsed thoroughly with water. Nevertheless, the solution does not need to be stored in the fridge and can be kept at room temperature for the duration of the experiment. The solid compound should be stored in a freezer. To make 0.1M diethyldithiocarbamic acid measure out 0.25956g of DDC solid and place into a 10mL volumetric flask. Make up 10mL of DDC using 0.2M Potassium phosphate buffer pH 7.6. Transfer solution into an appropriately sized universal container.

10M Sodium Hydroxide solution – This is a strong alkali used for quenching TAT and converting p-Hydroxyphenylpyruvate to p-Hydroxybenzaldehyde. p-Hydroxybenzaldehyde is readable at 331nm but the previous compound is not. There are severe hazards in using this strength of NaOH, including burns, irritations and ulcerations. It is also harmful as dust and becomes temporarily exothermic once prepared. Therefore, safety specs and plastic gloves should be worn throughout its preparation and use. To make 25mL of NaOH, measure out 10g of the compound into a beaker and transfer into a glass-measuring cylinder. Pour in distilled water to the 25mL mark and transfer the solution into an appropriately sized glass tube with a screw cap. As an extra control measure, you may wish to store the glass tube within a larger and thick screw-top glass container.

0.3M α-Ketoglutarate (2-oxoglutarate) solution – This compound is quite acidic, and a pH 7.6 is needed for it to be useful in this assay. Consequently, other solutions need to be made so that the pH can be altered to a useable level; an alkaline substance is necessary. Since you will be handling strong alkali, it is necessary to wear goggles and gloves. You need to make a decent amount of 1.5M NaOH, the instructions to make up 25mL are as follows: pipette 3.75mL of 10M NaOH that you’ve already prepared into a 25mL volumetric flask and fill with distilled water OR measure out 1.5g of NaOH and transfer into a 25mL v. flask. Since pH adjustments are tricky, you may need to make up an appropriate amount of acid solution – 1M HCl. To make up 10mL
of 1M HCl, measure 0.3646g of HCl into a 10mL volumetric flask and fill to the meniscus with water. 2M HCl was already prepared in the laboratory I worked in, therefore I just half-diluted it with distilled water. Measure out 2.1914g of a-Ketoglutarate into a 100mL conical flask. Place a small flea into the flask containing the a-Ketoglutarate and place it on a magnetic stirrer. Pour in from 15-20mL of 0.2M Potassium phosphate buffer pH 7.6 into the flask and activate the magnetic stirrer. Calibrate the pH measurer using pH7.0 buffer and measure the current pH of your solution. Gradually add the 1.5M NaOH solution into the flask until the pH of the resulting solution is 7.6. I needed to hold the flask containing the solution over the stirrer, so that the flea could properly spin. If the solution is more acidic than 7.6, compensate with small amounts of 1M HCl solution. Transfer the solution into a 50mL volumetric flask and fill to the meniscus with the 0.2M Potassium phosphate buffer. Transfer into a universal container and store in the fridge where it is stable over a long period.

Method of the modified Diamondstone assay – There are many variations to this assay that take place throughout the project. The following account is the basic method to perform this assay. Later in the method section, specific changes will be announced. Silica cuvettes should be used for this assay in all cases (1cm in width) and a UV-vis spectrophotometer is necessary to measure absorbance at this wavelength (331nm). The desirable ratio of enzyme and buffer in the 0.4mL aliquots will be determined first. Dr. PJ Evans of Cardiff University provided the neat (concentrated) enzyme preparation used to obtain a standard activity curve.

Add 2.3mL of tyrosine solution into a glass test tube and allow it to cool. Add the following to this to make the initial assay solution (2.9mL): 0.1mL pyridoxal-6-phosphate; 0.1mL diethyldithiocarbamic acid and 0.4mL solution of tyrosine aminotransferase + 0.2M potassium phosphate buffer. Incubate the test tube for 5 minutes at 37 °C; replace necessary solutions into the fridge. Start the initial reaction by adding 0.1mL of a-Ketoglutarate; scale the start time for the reaction and compensate when stopping it. For blanks add 0.2mL of 10M NaOH, spin on a whirlimixer for a number of seconds to denature the enzyme, and then add 0.1mL a-Ketoglutarate. Incubate the non-blank test tube(s) at 37 °C and stop the reaction after the exact desired time by adding 0.2mL of 10M NaOH solution. With the blanks - mix both together to form a homogenous sample of blanks (6.4mL), mix well and pour into separate cuvettes (approx. 3mL each). Wait 30 minutes for the secondary reaction to take place for the non-blanks, whilst incubated in a 37 °C water bath. Mix each test tube solution in a whirlimixer for a few seconds. Calibrate a UV-vis spectrophotometer at 331nm with one of the blank samples then add the alternative blank sample (they should be equal in absorbance); note down any
differences between the two. Measure the absorbance of any other non-blank samples at the same wavelength. Make adjustments to the non-blank readings so they are comparable to the blank you used to calibrated the spectrophotometer.

Initially, I determined the level of enzyme necessary in the assay. I found that a 1/4\textsuperscript{th} dilution was too powerful and the resulting absorbance was too high after 30 minutes. It went off the scale on the spectrophotometer. I also performed the assay using a 1/20\textsuperscript{th} and 1/40\textsuperscript{th} enzyme-buffer dilution with 15- and 30- minute time-points. I found that the 1/40\textsuperscript{th} dilution had given me the most reasonable measurement since the 30-minute absorbance was approximately twice that of the 15-minute absorbance. They were also substantial absorbance readings as they were not too close to 0.000. I had proven that the assay mixtures had worked and I had activity that was possibly related to the tyrosine aminotransferase because more p-Hydroxybenzaldehyde was measured.

Therefore, I performed the assay again to obtain a standard curve of which to base my results. I set up test tubes for 5-70 minutes at 5-minute increments and 2 blanks. I used 0.4mL of enzyme-buffer solution containing 1/40\textsuperscript{th} dilution of enzyme (0.2mL enzyme in 7.8mL 0.2M potassium phosphate buffer).

A repeat assay was made for 5-100 minutes at 5-minute increments and 2 blanks. Enzyme-buffer solutions were made using 0.2mL enzyme in 7.8mL 0.1M potassium phosphate buffer (or multiples using the same ratio). The standard curve was obtained using the standard modified Diamondstone assay.

The next stage of the assay was to ascertain whether or not leaving out certain components highlighted problems with the assay. Separate experiments were carried out without α-Ketoglutarate, pyridoxal-phosphate, L-tyrosine solution and diethyldithiocarbamic acid. For the experiment where I missed out tyrosine, 2.3mL of 0.2M potassium phosphate buffer pH 7.6 was used in its place. The other components, when missed out, were replaced with 0.1mL of the same buffer solution. A standard assay was performed with these so that they can compare with the original curve.

An assay was carried out without enzyme with a standard assay ran besides it. Therefore, 0.4mL of 0.1M potassium phosphate buffer was used instead of 0.4mL of enzyme-buffer solution. All of the results were read at 331nm.

Another assay was performed to measure pyridoxal-phosphate and diethyldithiocarbamic acid absorbance. Both were used in combination at 0, 10 and 20-minute points. The resulting solution comprised of 2.8mL of potassium phosphate buffer, 0.1mL PLP and 0.1mL DDC. The 0, 10 and 20 time-points were halted using 0.2mL 10M NaOH and mixed. A test tube was set up
using 2.9mL potassium phosphate buffer, 0.1mL PLP and 0.2mL 10M NaOH and another was set up using 0.1mL DDC instead of PLP. All the results were measured against a blank containing 3.0mL buffer and 0.2mL NaOH, at 331nm.

Yet another assay was performed using only enzyme, 0.1M potassium phosphate buffer and 0.2mL 10M NaOH. The enzyme concentrations were as follows: 100µL, 50µL, 10µL and 5µL. The solutions were made up to 3.0mL including buffer. Measurements were taking against a blank containing 3.0mL buffer and 0.2mL NaOH.

The final stage of this assay was to determine that twice as much enzyme should result in twice as much activity. For this, I set up experiments using 1/20th, 1/40th and 1/80th solutions of enzyme-buffer and performed the Diamondstone assay with 15- and 30-minute time-points. The protein solutions were stored in the freezer in separate plastic containers and were used in a protein estimation experiment described in the following sections.

Chemicals involved in the protein estimation assay – To make a determination of protein content in the samples of tyrosine aminotransferase, the following reagents need to be made up: Lowry reagent and ½ diluted Folin reagent. The ½ diluted Folin reagent was supplied by Dr. Evans. Lowry reagent requires a 50:1:1 ratio of 2% sodium carbonate NaCO₃ dissolved in 0.1M NaOH (v/v), 0.5% copper sulphate CuSO₄ (v/v) and 1% sodium-potassium tartrate (v/v). Therefore, to make at least 100mL of reagent, you used 100mL of NaCO₃, 2mL of CuSO₄ and 2mL of sodium-potassium tartrate. Lowry reagent needs to be made fresh daily but it’s components are stable at 4 C. ½ Diluted Folin reagent needs to be kept in the fridge and covered with aluminium foil because of its sensitivity to light.

Protein standard – Bovine serum albumin (BSA) is used as the protein standard. Make up 10mL of BSA solution by adding 0.1M potassium phosphate buffer to 0.01g BSA – using a volumetric flask.

Lowry reagent components – 1L of 0.1M NaOH needs to be made using 4g of solid in a volumetric flask and filled with distilled water. To make 1L of 2% NaCO₃, measure out 2g of solid and place into another 1L volumetric flask. Fill to the line with 0.1M NaOH you just made, transfer into a plastic container and refrigerator. To make 100mL of 0.5% CuSO₄, measure out 0.05g of the substance into a 100mL volumetric flask and fill with distilled water. This substance is described as an irritant so gloves should be worn whenever handling it as a solid or aqueous solution. Transfer into a container and store in the fridge. To make 100mL of 1% sodium potassium tartrate, measure out 0.1g of solid, transfer into a volumetric flask and fill with distilled water. This substance should also be stored in the fridge whenever it is not used. I found
that I had a large excess of leftover solutions after I had completed my project. Consequently, you may wish to make up half what is described here, using half the amount of substance if that is the case.

Protein assay using Lowry and Folin reagents – This assay is relatively easy to perform, and you should be able to do a substantial number of assays at one time. Make up your desired amount of Lowry reagent (i.e. 52mL) by measuring 50mL into an accurately marked measuring cylinder and pour it into a plastic labelled container. Pipette 1mL of CuSO4 solution and 1mL of potassium tartrate into the container and mix well. You need to make a protein-water solution totalling 0.5mL and for the standard protein estimation you use the solution of bovine serum albumin. Therefore, you may wish to measure the absorbance of 50µL of protein so you use 450µL of distilled water. Add 2.5mL of Lowry reagent to the protein-water sample into a test tube and incubate it for 5 minutes at 37 °C. For the next stage you must wear gloves because ½ diluted Folin reagent is an irritant. Add 0.25mL or 250µL of ½ diluted Folin reagent into the test tube and incubate for a further 15 minutes at 37 °C. This should give enough time for the proper colour to form, a greenish-blue. Mix the test tube’s contents in the whirlimixer then measure at 750nm using 1cm width silica cuvettes.

Measurements were taken in 10µL increments of BSA solution, from 0µL (+500µL water) to 200µL protein (+300µL water). A standard curve can be plotted from these results. Then I used the enzyme samples that I kept from the assays. I measured the protein content using the Lowry/Folin protein assay and samples with 25µL and 50µL of enzyme preparation. I got a reading for the 1/20, 1/40 and 1/80 dilutions of enzymes that I stocked in the freezer that were allowed to thaw.

Preparation of the hormone induced hepatocyte monolayers - Plates with a thin layer of rat hepatocyte cells were prepared and provided by Dr. P. J. Evans of Cardiff University. Dr. Evans inoculated 41 plate with 1mL of Leibovitz L-15 medium (pH 7.4) supplemented with 8.3mM glucose and 25mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid). 50µg/mL of gentamicin was added as an antibiotic to each of the plates, and 10% heat-inactivated newborn calf serum was also added. There were 8 plates set up in this way as the controls; no other solutions were added (2 control plates at zero hour, 1 control plate each for 1.5hr, 3hr, 4.5hr, 6hr, 7.5hr and overnight).

There were 6 plates for each of these conditions (1 for each time point): insulin and dibutyryl cyclic-AMP. There were 7 plates for the following conditions (1 for each 1.5hr, 3hr, 4.5hr and 6hr points, 2 for the 7.5hr time-point): dexamethasone; dibutyryl-cAMP and
dexamethasone; insulin, dibutyryl-cAMP and dexamethasone. There was in total 33 plates with hormones, each included the L-15 medium (including the supplements) and gentamicin antibiotic. 3mL of insulin (0.8µg/mL) was added to the 12 plates that required it. 3mL of 10⁻⁶M dexamethasone was added to the 21 plates requiring it. 3mL of 5x10⁻⁴M dibutyryl-cAMP was inoculated onto the 20 plates requiring it. Therefore, the plates intended for all 3 hormones were inoculated with 9mL of hormone solution, consisting of 3mL of each type.

For the given time-points described above, the plates were incubated at 37 °C. The medium was then removed from the plates so that only the hepatocyte monolayer remained. They were then covered with cling film to prevent condensation, and frozen down. Pictures were taken of several of the plates before they were frozen, to provide qualitative data.

Obtaining quantitative measurements of the hormone-induced hepatocytes – After the activity of tyrosine aminotransferase was determined, the activity of the hormone-induced plates was investigated. A 100mL solution of 0.2mM pyridoxal-phosphate (PLP) was made.

Set up for the assay - Dilute the 0.2M Potassium phosphate buffer pH7.6 to 0.1M using distilled water (a 1:1 dilution), mixing well. The molecular weight of PLP is 247.1; therefore measure 0.004942g of PLP in a weigh boat. Transfer the solid into a 100mL volumetric flask and add 100mL of 0.1M Potassium phosphate buffer. Mix the solution well and then transfer it into an appropriate container, cover with foil and store in the fridge until use.

To process a plate, I added 0.5mL of 0.2mM pyridoxal-phosphate solution onto the plate. Then, I scrapped off the cells from the plate using a glue scrapper (obtained from a stationary shop), which should collect in the pyridoxal-phosphate medium. I took up the cell-PLP medium with a pipette and expelled it into a small plastic tube, which should be kept on ice. Then I added another 0.5mL of pyridoxal-phosphate solution onto the plate, mopped up the remaining cells with the glue scrapper then took up the solution with a pipette, that I discharged into the same tube. The small tube were sealed and sonicated by Dr. Evans. Immediately after sonication, the tube is frozen until needed again. All the plates were processed and sonicated in one period, and placed in the freezer.

Firstly, I processed the tubes containing the 7.5hr duplicates, to establish the ideal enzyme concentration needed for the assay. The tubes containing the duplicate dexamethasone & dibutyryl-cAMP and dexamethasone, insulin and dibutyryl-cAMP were removed from the freezer and sat in a small beaker containing warm tap water. I made 4 enzyme solutions, a 1/8th and a 1/4th dilution of dexamethasone & dibutyryl-cAMP and dexamethasone, insulin & dibutyryl-cAMP. To make up a 1/8th enzyme-buffer solution, I extracted 250µL or 0.25mL of
enzyme preparation and transferred it into a small plastic container containing 1.75mL of 0.1M Potassium phosphate buffer pH7.6. This is enough to make 5 lots of 0.4mL enzyme-buffer solution. To make up a 1/4th enzyme-buffer solution, I used 500µL or 0.5mL of enzyme preparation and 1.5mL of 0.1M Potassium phosphate buffer.

‘Mock’ procedure using the duplicate hormone-induced enzyme preps – The same procedure is used as in the standard activity assay described earlier in this method. For each of the four enzyme situations, 2.3mL of tyrosine solution, 0.1mL PLP and 0.1mL of DDC was added into 4 test tubes labelled as 0, 0, 15 and 30, indicating time in minutes. 0.4mL of enzyme-buffer solution was added to each of the test tubes that were then incubated for 5 minutes at 37 °C and consequently adding 0.1mL of α-ketoglutarate activated the 15- and 30-minute tubes. The blank tubes (both 0 time-points) went through the same procedure as the other blank solutions described earlier. After 15 or 30 minutes had elapsed, respectively, 0.2mL of NaOH was added to quench TAT and convert its product, which occurred over a 30-minute period. The resulting solutions were measured spectrophotometrically at 331nm.

Final procedure in measuring enzyme induction using the Diamondstone assay – A separate assay is performed for all the conditions. 50µL of enzyme is used to make up the 0.4mL enzyme-buffer solution. All other components in the Diamondstone assay are included and measurements are made on the UV-vis spectrophotometer at a wavelength of 331nm. For each condition, prepare: 2 blanks, a 10- and a 20-minute time-point. Therefore, each condition uses its own blank. The 4½-hour plates were discarded.

Protein estimation was made of all the enzyme-hormone solutions. 25µL and 50µL samples of enzyme prep were used including 475µL and 450µL of water, respectively. Results were obtained by measuring each against a solution of blank made without enzyme, using 2 silica cuvettes. Absorbance is measured in 750nm.

Results

Once the reaction has started, tyrosine aminotransferase starts to produce p-hydroxyphenylpyruvic acid (pHPP). When NaOH is added, then the product converts into the readable p-hydroxybenzaldehyde (pHBA), giving an absorbance. Figure 1 indicates the expected trend for this reaction – the absorbance increases with the time of the reaction. The enzyme dilution was 1/40th and 0.2M potassium phosphate buffer pH7.6 was used in the enzyme-buffer solution. The trend shows that the reaction is linear for approximately 55 minutes.
where $A_{331} = 0.915$. The absorbance change was calculated by during the period between 20 and 40 minutes, in which the absorbance increased by $+0.355$ absorbance units. Therefore, the change was 0.01775 A units min$^{-1}$ (where A stands for absorbance). This is known as the primary assay.

However, the experiment was repeated and the results obtained are more reliable than the last (thence named the standard assay). This will be explained in the discussion section. Figure 1 shows the results for the repeat experiment, which shows an increasing absorbance over time meaning that more pHBA is made over the course of the reaction. The enzyme dilution was 1/40$^{th}$ and the buffer used was 0.1M potassium phosphate. The experimental curve stayed linear for a slightly short period of approximately 52 minutes where $A_{331} = 0.893$. Therefore, any results obtained that are higher than this absorbance level will need to be repeated at a diluted rate to ensure accuracy. The absorbance change in a 20-minute period was $+0.346$ (when the reaction was linear). The absorbance change is hence 0.0173 A units min$^{-1}$.

The next set of assays performed missed out certain components. A standard assay gave a change in absorbance reading of $+0.325$ over a 20-minute period; hence the absorbance change was 0.01625 A units min$^{-1}$. This is 93.9% that of the established assay and it is labelled as secondary assay in Table 1. Therefore, all the assays performed that day have been adjusted to allow for the discrepancy. Without the enzyme present, I found that the activity was 4.8% compared to the standard assay. The result I obtained from the lack of α-ketoglutarate was 6.4%. The activity without pyridoxal-phosphate was 35.0% of the original. The experiment lacking L-tyrosine had an activity of 9.2%. Finally, the experiment lacking diethyldithiocarbamic acid was 24.6% of the standard assay. These results show that missing out any of the components results in a reduced concentration of p-HBA and therefore all the components are essential for the reaction to take place efficiently and accurately. Table 1 (appendix) gives a detailed summary of the data above; the molar concentration of enzyme ($c$) is based on Beer’s law by dividing the unit change of absorbance (A) by the molar absorption coefficient of p-HBA ($\varepsilon$).

Figure 2 shows the absorbance of different concentrations of enzyme compared to each other against a blank consisting of only buffer and NaOH. The trend clearly shows a linear relationship between enzyme concentration and absorbance. Therefore, absorbance is affected by the amount of protein in the sample and that different blanks will be needed for different conditions, such as in the hormone-induced assays. However, the absorbance of the 1/40 dilution sample was relatively small - 0.030, so it will not significantly affect the results.
The absorbencies of pyridoxal-phosphate and diethyldithiocarbamic acid were compared to each other and separately, against a blank containing buffer and NaOH. The results show in Table 2 that they contribute to the absorbance separately and together, yet they are not additive together. They also cause an increase in absorbance after being left in the water-bath for periods of time, yet the results show that there is little evidence of increased activity over time. PLP has a slightly higher absorbance than DDC.

Table 3 indicates the effect of increasing the dilution of enzyme on the rate of absorbance. As expected, increasing concentration (reducing dilution) of enzyme results in an increased rate of reaction. Figure 3 indicates that an increase in bovine serum albumin increases steadily the light absorbance at 750nm. Based on this curve and the results in Table 3, I can compare my enzyme samples from previous experiments. Table 4 indicates that my 1/80\textsuperscript{th} and 1/40\textsuperscript{th} samples had the same amount of enzyme - 0.039 per mg protein, but my 1/20\textsuperscript{th} sample had slightly less – 0.033 per mg protein. These were calculated by the following formula:

\[
\frac{\text{Enzyme activity (A units/time)}}{\text{Protein content / mL}}
\]
Figure 2 – Absorbance vs. enzyme dilution indicates the affect of enzyme content on the absorbance in a given sample.

Figure 3 – Protein content versus absorbance using the Lowry protein assay and BSA.
Enzyme dilution and enzyme concentration | Enzyme / mg protein
--- | ---
1/80<sup>th</sup> | 0.039
1/40<sup>th</sup> | 0.039
1/20<sup>th</sup> | 0.033

Table 4 – Protein content of different samples of enzyme per mg. Results are based on those calculations from Table 3

From the initial results for the hormone-induced enzyme, I found that using 50µL of enzyme preparation per assay yielded the best results because my 30-minute reading was twice that of the 15-minute reading, which is expected. This is based on my duplicate 7.5h sample with dexamethasone and dibutyryl-cAMP. Using 100µL of enzyme per assay gave a high 15-minute reading and a reading off the scale for the 30-minute sample. Nevertheless, the 15-minute sample using 100µL of enzyme gave an absorbance twice that of the 15-minute sample with half the enzyme. Therefore, 50µL of enzyme will be used in all the assays of all the other enzyme preparations. The raw results are in Table 5 in the appendix. From this data, it was decided that 10- and 20-minute time-points were more suitable so that the readings are not off the scale set by the standard curve.

Microscopic analysis of the hormone-induced plates was undertook moments before they were frozen down. The following accounts are some examples of what was found in a few of the plates. The control plates showed that initially, the cells are mostly separate and there was little membrane interaction between them (see Figure 4). There weren’t any dead cells showing trypan staining. Figure 5 and 6 picture what the cells looked like after treated with insulin. Cells were closer together with a number of cells that had fused together and their membranes appeared to disappear. There were a few cells that were biologically dead and were stained with trypan dye; these are the cells on top of the monolayer. Figure 7 shows the cells after 4½ hours of dibutryl-cAMP addition, there were a lot of fused membranes and many cells were close. There were a number of dead cells on the top. Cells exposed to dexamethasone weren’t as close to each other and the membranes were rounded. However, there were a number of cells that had fused with one or two other cells. Some of them show cells containing many nuclei yet have no plasma membrane separating them. Figure 8 and 9 are the pictures that illustrate this.
Control study: Over the 26½-hour period, the samples showed little activity change over time compared to the zero hour control, the basal value. There was a very weak peak of 125% at the 3-hour point. During the overnight stage, there was a very slight dip in activity. There is no induction indicated by these results, shown on Figure 10.

Insulin study: The graph on Figure 11 shows some induction peaking at 278% of basal at the 3-hour point. After that point, the activity gradually decreases. There was a small increase at
the 7½-hour point. The graph indicates that the enzyme does not survive well during the overnight period, the level of activity dropped to 33% of the levels found at the zero hour control.

Dibutyryl-cAMP study: Figure 12 shows a steady increase of activity over time; there was relatively high induction compared to insulin. The graph shows no indication of reduced activity throughout the entire 26½-hour period, but theories about this will be discussed in the next section. All the other conditions showed a decrease in activity at the overnight point, dibutyryl-cAMP shows, on the contrary, that activity peaked after 26½-hours.

Dexamethasone study: There was a steady escalation of enzyme activity during the 0-7½ hour period. The induction peaked at 335% of basal, at the 7½-hour time-point. Like dibutyryl-cAMP, there were high levels of induction throughout the time period. However, judging from the shape of the graph in Figure 13, induction is higher in dexamethasone treated enzyme samples than of those treated with dibutyryl-cAMP. After a 26½-hour period, the level of activity drops to similar levels found in the control samples, a gradual decline after 7½ hours.

Dexamethasone and dibutyryl-cAMP studies: The graph in Figure 14 shows peaks and troughs. There was little change in activity after 1½ hours, after that point there was high levels of activity. There were peaks at the 3-hour and 7½-hour points, 320% and 604% respectively; there were steep increases before the peaks. Therefore, there was a very high level of induction compared to the other conditions and hence dexamethasone and dibutyryl-cAMP had an additive induction-effect on tyrosine aminotransferase. The activity gradually declined after 26½ hours. Nevertheless, the level after this period was still higher than all the other levels of induction in all the other conditions (357%).

Studies with all three hormones: The graph in Figure 15 has a similar style to that observed in the studies with both dibutyryl-cAMP and dexamethasone. However, there was not much change in the earlier enzyme preparations (1½- and 3-hour points) and there were not the steep peaks and troughs observed previously. After 3 hours, there was a strong increase in activity. Like in the samples with two hormones, the activity peaked at 7½ hours with an impressive induction level of 612% of the basal control, the highest level of induction obtained in these experiments. However, there were similar levels of induction displayed in the earlier assay using two hormones. This means that insulin had little additive effect with these two hormones. Nevertheless, the samples containing all three hormones showed the highest levels of induction compared to all the other conditions – therefore the addition of all three hormones gave the best induction of tyrosine aminotransferase.
Figure 10 – Relative activity of controlled enzyme

Figure 11 – Relative activity of enzyme + insulin

Figure 12 – Relative activity of enzyme + dibutyryl-cAMP
Figure 13 – Relative activity of enzyme + dexamethasone

Figure 14 – Relative activity of enzyme + dibutyryl-cAMP + dexamethasone

Figure 15 – Relative activity of enzyme + dibutyryl-cAMP + dexamethasone + insulin
Discussion

Despite the significant workload, I obtained comparative and indicative results. The assay method that I used worked well, and I gained a large number of results showing that hormones can indeed induce the enzyme tyrosine aminotransferase.

There were a few changes to the method, and one of those changes was the way that I originally made the two buffer solutions. I found that weighing such a large amount of solid proved difficult and that it would be easier if I weighed it in a large beaker instead; also I found that the powders didn’t go into solution very easily in the volumetric flaks. The benefit of measuring to a large beaker was that I could stir the mixture on the magnetic stirrer until it went into solution. I found before that I had to vigorously shake the flask. I had to make the buffer solution four times, three of which used this revised method and I found it less time consuming on those occasions.

The method of making tyrosine solution had also changed. Instead of making 500mL, I made 250mL regularly. This was because I found that the tyrosine doesn’t stay fresh for a long period of time, usually lasting only a few days on the hot-plate stirrer. Therefore, it was less wasteful to make ½ as much. I also changed the method so that it was more volumetrically correct. Instead of transferring the powder into a conical flask then adding the buffer measured using the volumetric flask, I made the solution by the method described above. I found that turning the initial heat up was essential for tyrosine to efficiently get into solution, but the temperature must be lowered after it had because otherwise the tyrosine broke down. I made approximately 6 lots of tyrosine solution during my project.

I made a small alteration to the method to make 2-oxoglutarate. The original method recommends that I made up 25mL of solution, but I found that this made it extremely difficult when doing pH adjustments. What happened was that the 1.5M NaOH made up most of the 25mL of solution. Making 50mL of solution was a lot more manageable, and a lot more buffer could be used in the solution. I found pH adjustments easier when working with a larger amount of solution. I had to make 2-oxoglutarate twice and both times I used the revised method.

I found during this project that pyridoxal-phosphate was necessary in the assay, otherwise the levels of p-Hydroxybenzaldehyde obtained was only a fraction. Pyridoxal-phosphate hence has an important role with tyrosine aminotransferase. It binds to the enzyme at the PLP-binding site, stabilizing the subunit. Hence, the enzyme would be able to function efficiently. Without PLP, the enzyme’s activity is greatly reduced and also unstable without its cofactor. The paper
that was written by Canellakis ZN. & Cohen PP. (1956) supports this hypothesis [10]. The cells treated with hormones were washed off their plates using a very low concentration of pyridoxal-phosphate solution – to stabilize the enzyme.

In addition, I found diethyldithiocarbamic acid (sodium salt) to be essential to the assay. This is explained by its activity against 4-hydroxyphenylpyruvate dioxygenase. Clearly without DDC, the assay showed poor levels of p-Hydroxybenzaldehyde. Therefore, the dioxygenase must be transforming the p-Hydroxyphenylpyruvate into homogentisic acid (the intermediate of the metabolic breakdown of tyrosine [25]), which is unreadable at 331nm in the UV-vis spectrophotometer. The paper by Lin et al. (1958) corroborates this [8]. It is assumed that the enzyme activity is the same in the assays without DDC but the product is being converted into something other than p-HBA. Unusually, Granner & Tomkins (1970) found that they did not need DDC in their experiments [26].

L-tyrosine and α-ketoglutarate obviously needed to be included in the assays because they were the substrates that were to be converted into products that were converted further to become readable. It is no surprise from the results that without them, only marginal levels of p-HBA were measured. The small amount of p-HBA found without tyrosine may be explained by the presence of small levels of tyrosine already present in the cells but tyrosine was a limiting factor. However, when including 2.3mL of L-tyrosine in the test tube, tyrosine is now in excess. I found in the standard assay that the reaction was still proceeding after 100 minutes meaning that neither tyrosine nor α-ketoglutarate had ran out so therefore neither were limiting factors at that point.

The low levels of activity without enzyme may be explained by the contamination of a small amount of bacteria that contained tyrosine aminotransferase. A certain level of bacterial or fungal contamination is to be expected even in the most carefully prepared experiments. Heilbronn et al. (1999) stated that *Klebsiella pneumoniae* have genes for this enzyme, but they revealed that there was only 13% homology of the bacterial TAT to eukaryotic TAT [27].

The reason why I used the repeat assay as the standard is because (a) the enzyme-buffer solution was made up using 0.2M potassium phosphate buffer pH7.6 for the primary assay, whereas all experiments beyond this point used 0.1M potassium phosphate buffer; this is because the concentrate of enzymes given by Dr. Evans were suspended in 0.1M potassium phosphate buffer that I supplied; and (b) readings from 0-100 minutes gave a more accurate picture of what was occurring and also I was able to determine when the assay was linear with more precision.
The plates treated with hormones showed various amounts of induction. As stated in the introduction, there is a great deal of literature with experiments on tyrosine aminotransferase. Some of those papers have differing results to each other - their hormone preparations behave differently. In this experiment, a monolayer culture of hepatocytes was used for each condition, because Evans and Mayer (1982) showed that the cells are metabolically stable for a longer period of time [28]. Therefore, I was able to leave the cells over a 26½-hour period without concern that the cells had become unstable. Evans also experimented with cAMP and dexamethasone in 1981 on TAT [29].

My results had shown that insulin does indeed have inductive effects on TAT, albeit the level of induction was quite small. The experiments by Pittner et al. showed that insulin does induce insulin, which supports the results that I have obtained. However, unlike mine, they found that insulin only increased activity by 34% but I found that my insulin treated plates induced TAT almost 3-fold. In their experiments they also used monolayer cell cultures [14]. Heaton et al. (1980) explained that insulin acts on the hepatocyte cells via “typical insulin receptors” and not via “the multiplication-stimulating activity receptor” that they studied. They determined this by using labelled insulin on hepatoma cells and they found insulin gave a 2-fold increase in TAT activity, similar to my own findings [30]. Gelehrter & Tomkins (1970) explained possible mechanisms for the 2- to 3-fold induction they found. They theorized that insulin acts on protein synthesis yet not on gene transcription. They also found that insulin does not sustain TAT induction, this supports my results of very low induction occurring at the 26½-hour point [31].

Dibutyryl-cAMP showed high levels of TAT induction and indicates that the induction lasts for more than 26½ hours. Pittner et al. also found high levels of induction by using an analogue of cAMP, between 2.6- and 3.4-fold. My induced enzymes may have lasted longer due to the fact that the cAMP analogue can withstand catabolic-attack by phosphodiesterase, which is present in hepatocyte cells [14]. Evans (1981) suggests that there are three possible mechanisms involved in the induction of TAT, namely enzyme activation, gene transcription and translation [29]. Hashimoto et al. discovered in 1984 that cAMP causes a 5-fold increase in hepatic TAT mRNA in which causes the transcriptional activation of the TAT gene effect [32]. This would explain why induction occurs in the presence of the secondary messenger analogue.

The above papers have stated that dexamethasone induces tyrosine aminotransferase such as the paper by Pittner’s group. I found that dexamethasone induced TAT by 3.35-fold after 7½ hours; Pittner stated that dexamethasone gave a 3.5-fold induction increase after 8 hours. Therefore both experiments show very similar findings [14]. However, I found that dexamethasone induction activity was not sustained after 26½ hours. Pittner didn’t find this; they
discovered that dexamethasone activity continues to rise for 24 hours [14]. Further experiments after 7½ hours would be needed to support their findings. A possible mechanism for this induction is because dexamethasone modulates EGF (epidermal growth factor) receptors in hepatic cells, consequently less tyrosine anabolism is required [33].

Together, I found that dexamethasone and dibutyryl-cAMP induced TAT additively. There was a 6-fold increase in activity after 7½ hours. Pittner’s paper supports my findings that both chemicals increased activity together. They theorize that their effects are independent of each other [14]. This would be supported by the theories that I made in the last two paragraphs. The two chemicals combined leads to a higher TAT mRNA concentration, reports Hashimoto [32], this paper also states that both chemicals have different mechanisms that make their induction additive. This would also make sense when considering the method - as the same amount of dexamethasone and dibutyryl-cAMP was in these plates as there were in the plates that consisted of dexamethasone or dibutyryl-cAMP alone.

Lastly, dexamethasone, dibutyryl-cAMP and insulin together showed similar induction levels as with just dexamethasone. Therefore, unlike dexamethasone and dibutyryl-cAMP, there was no additive effect of insulin with the other substances so the other substances may be actively inhibiting the action of insulin. Possibly by interacting directly with the insulin molecules or competition over the binding site of the insulin receptors. However, I concluded that this hormonal collection showed the best level of induction so insulin seems to have a small effect on TAT. Since the activity was not lower than the previous condition, I can also conclude that insulin does not have an antagonist or depressive effect on the mechanisms of dexamethasone and dibutyryl-cAMP. The findings of Pittner et al. would support my conclusions. They found that insulin had no significant effects on cAMP, possibly due to the resistance to phosphodiesterase mentioned earlier, since Pittner stated that insulin could stimulate phosphodiesterase that would antagonize cAMP [14]. My results to not mirror those found by Ho et al. (1981), who stated that induction of dexamethasone was largely inhibited by insulin. They theorized that induction of the enzyme appears in post-natal organisms when the levels of insulin in the liver decrease [34]. However, my experiment was not performed using postnatal rat hepatocytes but adult cells, this may explain why I didn’t find insulin antagonize dexamethasone. Ho et al. also mentioned this theory as they observed that insulin induces the enzyme in adult rat livers [34].

Unfortunately, no results were obtained during the 7½-hour time-points and the 26½-hour time-points. To improve this project, I would include experiments in between this period to determine when the induction levels do fall, and whether they peak after 7½ hours. More
accurate results might be obtained if I had set up the test tubes ready in the water bath before adding the various components. This may cause the reaction to immediately started when the enzyme is added, I may obtain more reliable 10-minute values then. This may occur because the temperature of the solution would already be 37 °C and therefore, any lag period may be reduced. Regrettably, I was unable to add the hormones to the monolayer plates myself because the area in which they were prepared was off-limits.

It would be interesting to extend this project into other areas. In the literature, the effect of actinomycin-D was encountered in many papers [14, 29, 31, 35]. Similar levels of actinomycin that are stated in these papers could be added to the Leibovitz medium before addition of hormones. Using the same procedure, the induction of aminotransferase could be measured and compared to the results that I have obtained in this project. Therefore, I would be able to conclude whether or not actinomycin-D blocks any of the hormones, notably dibutyryl-cAMP [29].

Acknowledgements

I wish to thank Dr. PJ. Evans of Cardiff University for providing guidance throughout this project and for providing me with the chemicals needed to perform the various assays. Pyridoxal-5-phosphate and diethylidithiocarbamic acid (sodium salt) were manufactured by Sigma-Aldrich. Sodium-potassium tartrate, L-tyrosine and cupric sulphate were manufactured by BDH Chemicals Ltd. Fisher Scientific UK manufactured the chemicals Potassium dihydrogen orthophosphate and di-Potassium hydrogen orthophosphate. α-Ketoglutarate was originally from Prolabo. All the figures were made with the aid of Jasc Photoshop Pro v6.02; figures 4-9 were sharpened by JPSP and the edge enhancements were made to figure 6 using this tool.

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Appendices

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Absorbance units min^{-1}</th>
<th>Molar concentration (µg/mg/min)</th>
<th>Activity based on the standard (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>0.0173</td>
<td>0.694</td>
<td>100.0</td>
</tr>
<tr>
<td>Primary</td>
<td>0.01775</td>
<td>0.713</td>
<td>102.6</td>
</tr>
<tr>
<td>Secondary</td>
<td>0.01625</td>
<td>0.653</td>
<td>93.9</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>0.000083</td>
<td>0.003</td>
<td>4.8</td>
</tr>
<tr>
<td>Minus α-ketoglutarate</td>
<td>0.0011</td>
<td>0.044</td>
<td>6.4</td>
</tr>
<tr>
<td>Minus L-tyrosine</td>
<td>0.0016</td>
<td>0.064</td>
<td>9.2</td>
</tr>
<tr>
<td>Minus PLP</td>
<td>0.00605</td>
<td>0.243</td>
<td>35.0</td>
</tr>
<tr>
<td>Minus DDC</td>
<td>0.00425</td>
<td>0.171</td>
<td>24.6</td>
</tr>
</tbody>
</table>

Table 1 – Activity comparisons between the various assays expressed as the % activity of the standard assay determined by the modified Diamondstone assay.

<table>
<thead>
<tr>
<th>Assay condition</th>
<th>Time left in water-bath before addition of NaOH (mins)</th>
<th>Absorbance at 331nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP only</td>
<td>0</td>
<td>0.169</td>
</tr>
<tr>
<td>DDC only</td>
<td>0</td>
<td>0.159</td>
</tr>
<tr>
<td>PLP+DDC</td>
<td>0</td>
<td>0.168</td>
</tr>
<tr>
<td>PLP+DDC</td>
<td>10</td>
<td>0.366</td>
</tr>
<tr>
<td>PLP+DDC</td>
<td>20</td>
<td>0.377</td>
</tr>
</tbody>
</table>

Table 2 – Shows the effect of the assay components on the absorbance together and separately. It also shows the effect of leaving the samples in the water-bath after the 5 minute incubation period (time left signifies total time minus the 5-minute initial incubation stage).

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Absorbance change/min (A units)</th>
<th>Enzyme activity per mL</th>
<th>Protein content in 50µL enzyme (µg)</th>
<th>Protein content (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/80th</td>
<td>0.0036</td>
<td>0.72</td>
<td>11.5</td>
<td>18.4</td>
</tr>
<tr>
<td>1/40th</td>
<td>0.0094</td>
<td>0.94</td>
<td>30.0</td>
<td>24.0</td>
</tr>
<tr>
<td>1/20th</td>
<td>0.0138</td>
<td>0.69</td>
<td>51.5</td>
<td>20.6</td>
</tr>
</tbody>
</table>

Table 3 – Calculations of absorbance change, enzyme activity and protein content of my samples.
<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Dex + Db-cAMP 50µL</th>
<th>Dex + Db-cAMP 100µL</th>
<th>Ins + Dex + Db-cAMP 50µL</th>
<th>Ins + Dex + Db-cAMP 100µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.462</td>
<td>0.985</td>
<td>0.611</td>
<td>1.183</td>
</tr>
<tr>
<td>30</td>
<td>0.979</td>
<td>+2.000</td>
<td>+2.000</td>
<td>+2.000</td>
</tr>
</tbody>
</table>

Table 5 – Comparisons of Absorbance, λ = 331nm (A units) of 4 different enzyme conditions. Absorbencies over 2.000 indicate that it went off the scale. Dex stands for dexamethasone; Db-cAMP for dibutyryl-cAMP; Ins for insulin.