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# Distribution of Adenylyl Cyclase and Guanylyl Cyclase in Rat Tissues

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ABSTRACT. The specific activities of adenylyl and guanylyl cyclases, the cAMP and cGMP phosphodiesterase of rat kidney, liver, heart and brain were examined. Of all tissues, kidney has the highest adenylyl cyclase specific activities by mean (± SD) of (145 ± 14 pmol/min/mg protein) followed by that of heart (113 ± 21 pmol/min/mg protein), brain (101 ± 8 pmol/min/mg protein) and liver (92 ± 9 pmol/min/mg protein). The specific activity of particulate guanylyl cyclase was the highest in liver (26 ± 1 nmol/min/mg protein), but the soluble form predominated in kidney (40 ± 2 nmol/min/mg protein). In contrast to the other tissues examined, brain showed relatively high cAMP and cGMP phosphodiesterase activities. The kinetic properties of the adenylyl cyclases and cAMP phosphodiesterases were also investigated. The optimum pH for both types of activity was found to be at 7.4. Subcellular fractionation of the kidney to locate adenylyl cyclase activity revealed that both the mitochondrial and the microsomal fractions had higher specific activities than that of the nuclear fraction. Studies on the tissue distribution of cyclic nucleotide PDE activity as well as adenylyl and guanylyl cyclase showed that it is widely distributed in intra and extracellular and they have an important role in signal and nucleotide transdaction.

## Introduction

The physiological role of cAMP and cGMP as “second messengers” means that the relative concentration of these nucleotides in individual tissues is of prime importance in metabolic regulation. The intracellular levels of cAMP and cGMP depend largely on the relative activities of the biosynthetic enzymes, adenylyl cyclase and guanylyl cyclase, and on the 3′,5′-cyclic nucleotide phos-

phodiesterases, which hydrolyse cyclic nucleotides to their corresponding 5′-monophosphates[1].

Mercapide *et al.*[2], reported that they separated four families of PDEs from pig aorta: PDE1 (calcium-calmodulin activated), PDE3 (cGMP-inhibited), PDE4 (adenosine 3′,5′-cyclic monophosphate specific) and PDE5 (cGMP-specific). Within this PDE complement, PDE1 and PDE5 mostly contributed to the hydrolysis of cGMP both in the presence and absence of calcium-calmodulin. Hermsdorf *et al.*[3] reported that 67% of total cAMP-PDE activity in cultured rat hepatocytes could be detected in the cytosol, 15% in plasma membrane, 15% in dense vesicle and 3% in endoplasmatic reticulum fractions. Up to 84% of the PDE activity of the cytosol is represented by the raliprame-sensitive PDE4. Coudray *et al.*[4], studied the characteristics of phosphodiesterases present in brown adipose tissue (BAT) of *zucker* rat pups to determine whether the capacity for degradation of cyclic nucleotides was affected by fatty genotype. Regardless of the genotype, PDE2-4 contributed to total PDE activity. The PDE3 activity equalling the sum of PDE2 and 4 activities. Moos *et al.*[5], (1996) improved affinity support for the purification of adenylyl cyclase prepared from 7-desacetyl-7-aminoethyl-aminocarbonyl forskolin. This analog allows convenient synthesis of an affinity matrix that is chemically stable, with standing repeated use for up to two years, and efficient, yielding purifications of adenylyl cyclase from solubilized bovine brain membranes of 2000-6000 fold in a single step. Bocanera *et al.*[6], studied the partial characterization of the enzyme guanylyl cyclase in bovine thyroid, it comprises around 79% of total activity, while the other is particulate. The kinetics of the enzyme showed a Michaelis type kinetics for the soluble enzyme with a Km of 0.37 nM, whereas the particulate GC showed a positive allosteric behavior with a S0.5 of 0.214 mM. Recently, Sinclar *et al.*[7], reported that in mammals tissues there are two types of adenylyl cyclase synthesize cAMP, a ubiquitous family of transmembrane isoforms regulated by G-proteins in response to extracellular signals, and an isolated soluble enzyme insensitive to heterotrimeric G protein modulation. The soluble adenylyl cyclase (sAC) is detected in almost all tissues examined.

The main aim of the present study was to compare the specific and the relative activities of adenylyl cyclases and guanylyl cyclase in various rat tissues. The investigation was limited to the measurement of the activities of cAMP phosphodiesterase (cAMP-PDE), cGMP phosphodiesterase (cGMP-PDE), guanylyl cyclase, and adenylyl cyclase in partially purified extracts. This was done to minimize the effect of changes in activity that could be caused by purification procedures. The work also aimed to investigate the kinetic properties of cAMP phosphodiesterase and adenylyl cyclase in the environment of a tissue extract.

## Materials and Methods

### *Animals and Chemicals*

All biochemicals including cyclic nucleotides and column chromatography materials were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Radiochemicals including [2-$^3$H] adenosine-5′-triphosphate (ammonium salt) and [8-$^3$H] guanosine-5′-triphosphate (ammonium salt) were obtained from Amersham International Plc (Amersham, Buckinghamshire, U.K.). The high efficiency ‘Ready Safe’ scintillation cocktail, containing phenylxylylethane surfactant, used for aqueous samples was obtained from Amersham. The source of mammalian tissues used in this study was male Wistar rats. Their average weight were (250-350 g), and randomly selected from a rat colony bred from animals obtained in 1976 from Olac Ltd, U.K. in the experimental animal unit of King Fahad Medical Research Center. They were housed in an air-conditioned room at 24ºC and maintained on a 12 hr dark/light cycle. Animals were fed on standard food produced by Grain, Sails and Flour Mills Organization, Western Province, Saudi Arabia.

### *Extraction of Phosphodiesterase*

The phosphodiesterase (PDE) extraction method of Newton and Salih[8] was used with minor modifications. Tissues were rapidly removed, placed on ice, and then cut into small pieces, placed and then homogenized in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 6 mM 2-mercaptoethanol. Homogenization, in a Potter-Elvejhem homogenizer, was for a total of 3 min, in three 1-min bursts. For each of tissue, 9 ml of buffer were used. The resultant homogenate was centrifuged at 3000 g for 10 min at 2ºC. The supernatant was 100% saturated with ammonium sulphate and stirred for 1 hr at 4ºC. The precipitate was collected by centrifugation for 10 min at 1200 g and redissolved in 2-3 ml of 50 mM Tris-HCl (pH 7.4). After overnight dialysis at 4ºC against 50 mM Tris-HCl (pH 7.4) the non-dialysable fraction was centrifuged for 10 min at 6000 g to remove denatured protein. The supernatant was assayed for phosphodiesterase activity as described below.

### *Determination of Protein*

Protein was determined by the method of Bradford[9].

### *Extraction of Guanylyl Cyclase*

*a) Soluble form*

Weighed tissue samples were placed on ice, but into small pieces and homogenized in ice with ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM

theophylline and 6 mM 2-mercaptoethanol (1 mg tissue weight: 6 ml buffer) for a total of 3 min in 1 min burst. The homogenate was centrifuged at 12000 g for 10 min and the supernatant taken for assay of soluble guanylyl cyclase activity and protein content.

*b) Particulate form*

The pellet from the homogenate, described above, was washed twice, by re-suspending it in a similar volume of 50 mM Tris-HCl (pH 7.4) buffer containing 10 mM theophylline and 6 mM 2-mercaptoethanol. After centrifuging at 12000 g for 5 min, the washings were discarded and the resultant precipitate was resuspended in 2 ml of the same buffer, and homogenized for 10 sec. The homogenate was assayed for particulate guanylyl cyclase activity and protein content.

### *Extraction of Adenylyl Cyclase*

Weighed tissue samples were placed on ice and cut into small pieces, and homogenised in ice-cold 45 mM Tris-HCl (pH 7.4) containing 6 mM 2-mercaptoethanol, 8 mM theophylline and 0.25 M sucrose for a total of 3 min, in three 1 min bursts, at maximum speed (1500 rpm). For each g of tissue, 9 ml of buffer were used. The homogenate was centrifuged at 3000 g for 10 min at 2ºC. The supernatant was recentrifuged at 150000 g for 40 min at 2ºC. The final precipitate was resuspended in 2-3 ml of 45 mM Tris-HCl (pH 7.4) containing 6mM 2-mercaptoethanol and 8 mM theophylline. It was then divided into two parts, one was assayed for adenylyl cyclase activity and the other was used for determination of protein content.

### *Cyclase Nucleotide Phosphodiesterase Assay*

The specific and the total activities of partial purified cAMP-PDE and eGMP-PDE preparations from liver, kidney, heart and brain were determined. Using the malachite green method of Baykov *et al.*[10], samples (100 µl) of the partial purified enzyme preparation were added to incubation mixtures containing 100 µl of 5 mM cAMP or cGMP and 100 µl of 4 mM $MgSO_4$, and made up to a final volume of 500 µl with Tris-HCl buffer. The mixtures were incubated for 10 min at 37ºC and the assay reaction was terminated by heating in a water bath at 90ºC for 2 min. After cooling to room temperature, 100 µl or a 1 mg/ml solution of snake venom nucleotidase was added and the mixture was incubated at 37ºC for a further period of 1 hr. This reaction was stopped by boiling for 2 min and the incubation mixture was then centrifuged at 8700 g for 20 min. A 600 µl sample of the supernatant was taken and mixed with 150 µl of malachite green. After 10 min, the absorbance was read at 630 nm. The sen-

sitivity of the assay was limited by the control readings ('boiled enzyme' and 'no enzyme' controls) and was carried out in triplicate. Boiled enzyme was prepared by placing the extract in a water-bath at 90ºC for 3 min.

### *Adenylyl Cyclase Assay*

The activity of adenylyl cyclase was assayed by Alvarez and Daniels method[11] with minor modifications.

The adenylyl cyclase reaction was initiated by adding 75 µl of enzymic extract in 1.5 ml capped Eppendorf tubes kept on ice. The total volume of the reaction medium was 200 µl. It contained 0.1 mM adenine to decrease the specific radioactivity of nonphosphorylated of ATP generated during the reaction, 4 mM $MgSO_4$, 2 mM cAMP, 0.1 mM GTP, 45 mM Tris-HCl buffer (pH 7.4) containing 10 mM theophylline and 6 mM 2-mercaptoethanol, 0.5 mM (1.0 µCi) [2-$^3$H] ATP. Finally, 2 µl ethanol were added to increase the membrane fluidity of the homogenate[12]. All components were mixed gently and the tubes were incubated at 37ºC for 10 min and the reaction was stopped with 20 µl of 2.2 M HCl. The contents of the tubes were mixed gently and centrifuged at 8700 g for 10 min. The total supernatant from each tube was pipetted on to a separate column. Each column contained 1.3 g wet acidic alumina (acidic alumina was soaked in water for 2 min and the water was decanted to remove the small particles with it). The supernatant was allowed to flow into the alumina and then the column was washed through with 8 ml of 5 mM HCl followed by 1 ml of 0.1 M ammonium acetate. The total cyclic AMP was eluted with 3.5 ml of 0.1 M ammonium acetate, and collected into scintillation vials. To the eluate in the vials, 6.5 ml of Ready Safe Scintillation Cocktail was added, and tubes were counted in a β-scintillation counter.

### *Guanylyl Cyclase Assay*

The activity of guanylyl cyclase was determined in an assay mixture consisting 1 mM GTP containing 80,000-100,000 dpm of [8-$^3$H] GTP, 4 mM $MnCl_2$, 1 mM theophylline (phosphodiesterase inhibitor), 0.02% (w/v) bovine serum albumin to stabilize the enzyme activity[13], 1 mM $NaN_3$ (to inhibit nucleotide triphosphatase), 20 mM creatine phosphate, and 150 U/ml creatine phosphokinase (to regenerate ATP). The components were made up in 50 mM Tris-HCl (pH 7.4) containing 10 mM theophylline and 6 mM 2-mercaptoethanol. Reactions were initiated by the addition of 30 µl enzyme extract and the mixture was incubated at 37ºC for 10 min. Reactions were terminated by standing the mixture for 3 min in a water-bath at 100ºC. The mixture was centrifuged at 8700 g for 20 min and the resultant supernatant was applied to an acidic alumina column. Preliminary experiments showed that the Alvarez and Daniels[11] elution procedure could be

modified for use in determining cGMP. The modification involved washing the column with 5 ml of 0.03 M HCl, followed by 14 ml of 0.1 M ammonium acetate. The eluent (14 ml), which contained the cyclic GMP, was collected and 1 ml samples was added to vials each containing 5 ml of scintillation cocktail, which were counted in a β-scintillation counter.

### *Subcellular Fractionation Method*

The subcellular fractionation method of Birnie[14] was used. All assays were carried out in triplicate and both boiled enzyme and no-enzyme controls were used. Data are the mean of triplicate determinations from each of 4 rats. The error bars represent ± SD.

## Results and Discussion

Figure 1 shows the specific activity and the total activity of cyclic nucleotide phosphodiesterase (PDE) in each tissue. The highest specific activity was seen in brain tissue. This could be due to the presence of either a single, relatively specific, phosphodiesterase or, more likely, the existence of several phosphodiesterase of different specificity. The specific activity of phosphodiesterase in heart muscle tissue was the lowest of those tissues examined. This could be because of PDE loses its activity during extraction, either because an activity factor is removed or because 'proteolytic clipping' reduces the catalytic activity[15]. This result is similar to that of Fougier *et al.*[16,17]. This is compatible with the physiological role of cAMP level in the brain that mediates synaptic transmission, at certain types of synapses[18]. Regarding total activity of cAMP-PDE, as shown in Fig. 1 brain has the highest where liver shows a lower total activity than brain, kidney or heart. The total activities of kidney and heart were similar to each other.

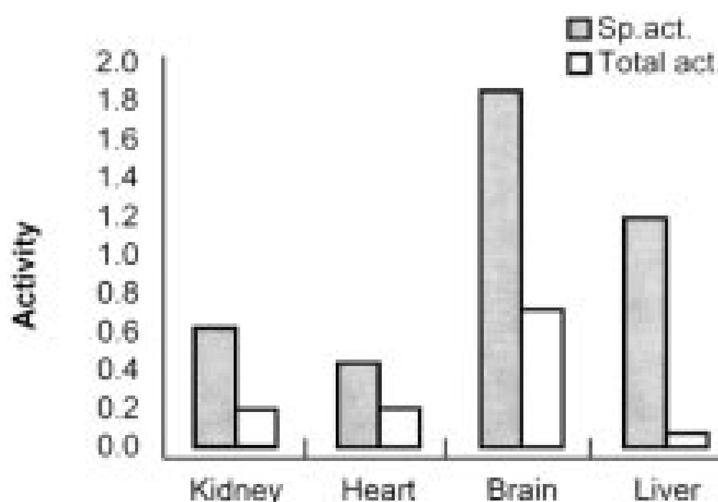


FIG. 1. Specific (nmol/min/mg protein) and total (nmol/min/g tissue) activities of cAMP-PDE in rat tissues. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

The effect of substrate concentration on cyclic AMP-PDE activity was examined with cAMP as the substrate. Concentrations ranging from 0.5 mM to 5 mM were examined using a fixed amount of the enzymatic extract in the reaction mixture. As shown in Fig. 2, the enzyme displayed a classic kinetic profile with the velocity increasing towards an asymptotic value as the concentration increases.

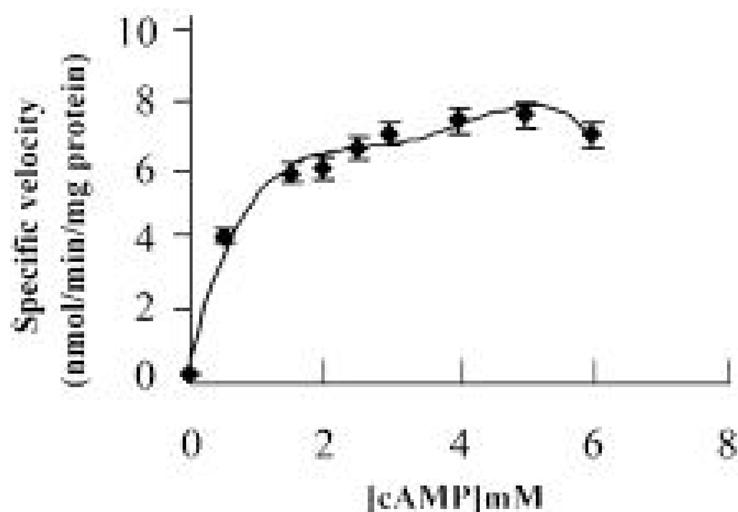


FIG. 2. Effect of increasing substrate concentrations on cAMP-PDE activity from rat liver. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

A Lineweaver-Burk double reciprocal plot of velocity versus cAMp concentration yielded a straight line intercepting the Y-axis at $1/V_{max}$ and the X-axis at $-1/K_m$ (Fig. 3) indicating Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values were 0.53 mM and 8.0 nmol/min/mg protein, respectively. This agrees with the results of Pichard & Cheung[19].

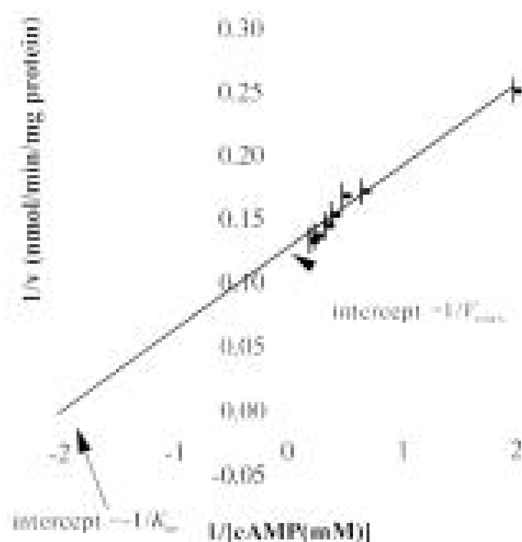


FIG. 3. Lineweaver-Burk plot of cAMP-PDE of rat liver extract. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

The specific activity and the total activity of cGMP phosphodiesterase from different tissues were examined and the results are shown in Fig. 4. They show that the brain extract has the highest specific activity whereas the activity of heart extract has the lowest. This is similar with cAMP-PDE (Fig. 1). Cyclic AMP phosphodiesterase from extracts of brain, liver, kidney and heart was found to significantly higher than the activity of cGMP phosphodiesterase from the same tissue. The results of specific activity shown in Fig. 4 in agreement with those of Campbell and Oliver[20].

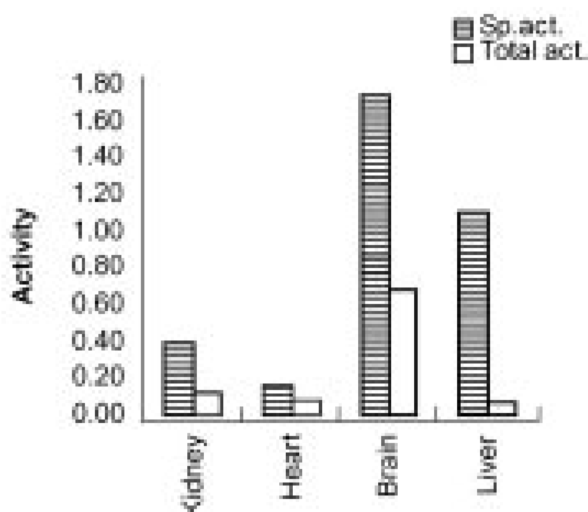


Fig. 4. Cyclic GMP-PDE specific (nmol/min/mg protein) and total (nmol/min/g tissue) activities in different tissues. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

### *Determination of Guanylyl Cyclase Activity*

The specific activity of guanylyl cyclase in both the pellet and the supernatant obtained from crude homogenates of liver, kidney, heart and brain are shown in Fig. 5. Kidney reflects the highest specific activity and has both forms of guanylyl cyclase, *i.e.* the particulate, and the soluble. Of these, the soluble form has the most activity. Both heart and brain are similar to one another in respect to their relative particulate and soluble activities. They show much lower specific activities than the other tissues and with both there is higher activity in the pellet than in the supernatant. These results agree with those reported by Hardman and Sutherland[21]; and White[22] for rat heart. Liver is different from the other tissues in that it only contains significant guanylyl cyclase activity in the particulate fraction and also has a higher specific activity than that of any of the other tissues examined. The guanylyl cyclase activity was observed the highest in the supernatant fraction of kidney, while there was no activity at all in the supernatant fraction of liver. The apparent absence of guanylyl cyclase activity from supernatant fractions of liver could be due to the enzymic hydroly-

sis of cGMP during the incubation period[13] and that subsequently to the physiological role of liver.

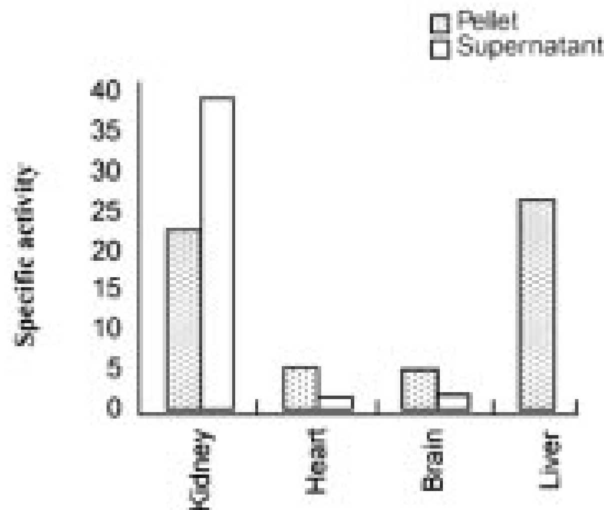


FIG. 5. The specific activity (nmol/min/mg protein) of guanylyl cyclase from different rat tissues. A modification of the method of Alvarez and Daniels (1992) was used. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

Figure 6 shows that the total guanylyl cyclase activity of the kidney supernatant fraction is higher than that of either heart or brain. The total activities of the supernatant guanylyl cyclase from heart and brain are however very similar. With respect to the particulate fraction, that from heart has a higher total activity than that from kidney, brain or liver. The total activity of particulate guanylyl cyclase from liver is the lowest of the tissues examined and this contrasts with the specific activity of the particulate guanylyl cyclase activity of liver. In order of decreasing total activity for particulate guanylyl cyclase, the tissues fall into the sequence, heart, kidney, brain and liver.

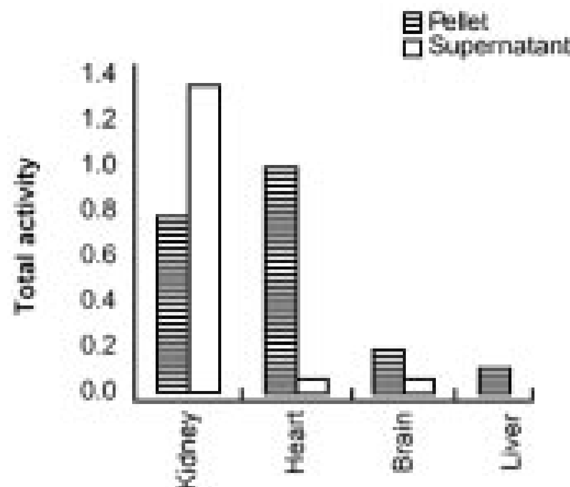


FIG. 6. The total activity (nmol/min/g tissue) of guanylyl cyclase from different rat tissues. A modification of the method of Alvarez and Daniels (1992) was used. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

### *Determination of Adenylyl Cyclase Activity*

Figure 7 shows that the highest specific activity of adenylyl cyclase was obtained in kidney, it was 1.3 fold, 1.4-fold, and 1.6-fold, higher than the corresponding enzymic activity in heart, brain and liver, respectively. This relates to the function of the kidney in preserving the internal environment of the cells, *i.e.* it maintains water balance, pH, ionic equilibrium, and fluid osmotic pressure. Due to decreasing adenylyl cyclase specific activity, the tissues examined fall into the order kidney (145 ± 14 pmol/min/mg/ protein), heart (113 ± 21 pmol/min/mg protein), brain (101 ± 8 pmol/min/mg protein), and liver (92 ± 9 pmol/min/mg protein). The variation in the activity of adenylyl cyclase from one tissue to another probably reflects their different physiological functions and may also reflect the extent to which various ATP-utilizing contaminating enzymes are present.

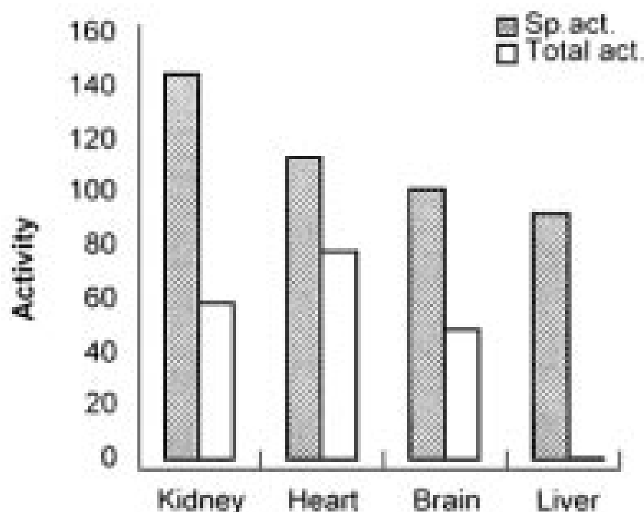


FIG. 7. Specific activity (pmol/min/mg protein) and total (pmol/min/g tissue) activities of adenylyl cyclase from different rat tissues. The assay method of Alvarez and Daniels (1992) was used with minor modification. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

Bär and Hahn[23] mentioned that the specific activity of adenylyl cyclase in rat liver is 2 pmol/min/mg protein while Johnson[24] notified the specific activity for the same tissue is 9 pmol/min/mg protein. The present results, (Fig. 7), show relatively higher specific activities than others which is attributable to the efficiency of the modified Alvarez and Daniels[11] method and the presence in partial purified membrane preparations of stimulatory factors, that is absent in purified preparations. Another contributory factor was almost certainly is the use of an ATP-regenerating system.

Determination of the total activity of adenylyl, (Fig. 7), indicates that heart has the highest and liver has the lowest total activity. So, in decreasing order to

total activity of adenylyl cyclase in the tissues examined, the sequence is, heart, kidney, brain and liver.

Newton and Brown[25] reported that cGMp levels in mammalian tissues are 10-100 fold lower than those of cAMP, a finding compatible with the enzymic activities measured in this study. It should be noted that the formation of cAMP and cGMP are under separate hormonal and metabolic control[26].

The effect of varying substrate (ATP) concentrations between 0.1 mM and 0.7 mM, with a fixed adenylyl cyclase concentration, was investigated in order to determine $V_{max}$ and $K_m$ for the adenylyl cyclase. As Fig. 8 shows, that the optimal concentration of ATP, as a substrate, is 0.5 mM. Increasing the substrate concentration above 0.5 mM caused a fall in activity (Fig. 8). This indicates substrate inhibition at higher concentrations. A Lineweaver-Burk double reciprocal plot of velocity (1/v) versus substrate concentration (1/[S]) yielded a straight line (Fig. 9), and indicated that the enzyme follows Michaelis-Menten kinetics. Values of 1.25 mM and 313 pmol/min/mg protein, are obtained for $K_m$ and $V_{max}$, respectively.

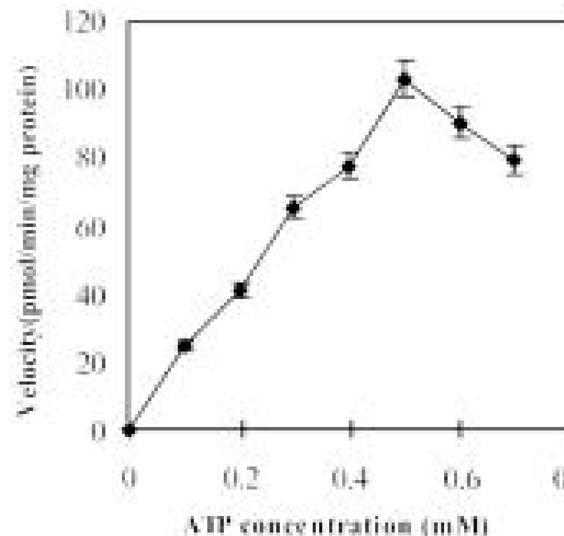


FIG. 8. Effect of substrate ATP concentration on adenylyl cyclase activity (pmol/min/mg protein). Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

Since rat kidney has the most active adenylyl cyclase of the tissues examined in this study, it was used for an investigation of the subcellular distribution of the enzyme. Table 1 shows that the specific activity of the adenylyl cyclase of mitochondria is only slightly different from that of the microsomal fraction. Both the mitochondrial and microsomal fractions have a higher specific activity, however, than that of nuclei. This is in agreement with the findings of Seraydarian and Mommaerts[27], and with those of Rabinowitz *et al.*[28].

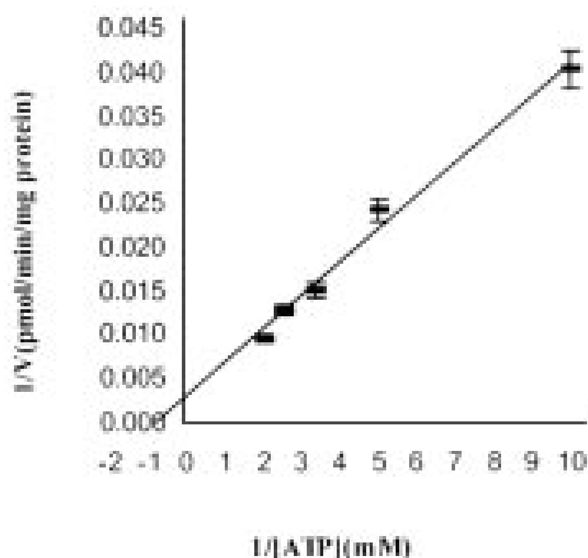


FIG. 9. Lineweaver-Burk plot of adenylyl cyclase activity from rat kidney. Data are the mean of 6 rats; each tissue extract was assayed in triplicate ± SD.

TABLE 1. Specific activity (pmol/min/mg protein) of adenylyl cyclase from subcellular fractions of rat kidney. Sucrose-EDTA buffer was used at pH 7.4

| Subcellular fractions | Specific activity (pmol/min/mg protein) |
|---|---|
| Homogenate | 116 ± 26 |
| Nuclei | 147 ± 32 |
| Mitochondria | 186 ± 36 |
| Microsomes | 188 ± 40 |
| Cytoplasm | 8 ± 2 |

Examination of the cytosol in this study showed no significant adenylyl cyclase activity. The result is in agreement with the findings of other workers[27 & 28]. Those workers concluded that the adenylyl cyclase system exists primarily in plasma membranes but may also exist and function in other cellular membranous structures. The present results with rat kidney support this conclusion.

## *Acknowledgement*

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# توزيع إنزيمات أيض النيوكليوتيدات الحلقية في أنسجة الجرذ

**جلال الدين خان و وديعة صالح بكر**
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*المستخلص.* إن الدور الفسيولوجي الذي تقوم به النيوكليوتيدات الحلقية مثل cGMP & cAMP كناقلات ثانوية في أنسجة الحيوانات الثديية ، دفعنا لدراسة إنزيماتها الحلقية والتي تقوم بعملية تنظيم لتركيز هذه النيوكليوتيدات في الجسم . فتكون النيوكليوتيدات الحلقية عن طريق إنزيمي Adenylyl cyclase و Guanylyl cyclase وتحلل هذه النيوكليوتيدات عن طريق إنزيمات خاصة أخرى مثل cAMP phosphodiesterase و cGMP phosphodiesterase لها أهمية قصوى في إعداد هذا البحث .

وقد تم تناول عينات من أنسجة الكلي ، الكبد ، القلب ، والمخ من الجرذ وتم قياس تركيز ونشاطية هذه الأنزيمات في هذه الأنسجة ، وقد دلت النتائج على أن نشاطية إنزيم Adenylyl cyclase في الكلى أعلى من القلب يليه المخ ثم الكبد ، أما بالنسبة لإنزيم Guanylyl cyclase غير الذائب فإن نسبة نشاطه في الكبد أعلى من نسبة نشاطه في الأنسجة الأخرى (القلب ، الكلى ، المخ) أما ا لذائب منه فنشاطه أعلى في الكلى ، كما تبين لنا من الدراسة أن إنزيمات cGMP و cAMP phosphodiesterase لها نشاطيه ملحوظة في المخ يزيد بكثير عن بقية الأنسجة الأخرى .

ومن خلال دراسة الخواص الحركية لكل من cAMP phosphodiesterase و Adenylyl cyclase وجد أن الأس الهيدروجيني المثالي (optimum pH) هو ٧,٤ .

كما تم أيضًا تحديد توزيع إنزيم Adenylyl cyclase في داخل الخلية الكلوية في الجرذ ، فوجد أن تركيز الإنزيم في جسيمات الميتوكوندريا والكروسوم أكثر منه مقارنة بالنواة . نتائج الدراسة ومقارنتها بالنتائج الأخرى في متن البحث .