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OROTIDYLATE DECARBOXYLASE FROM HUMAN BRAIN[1)]

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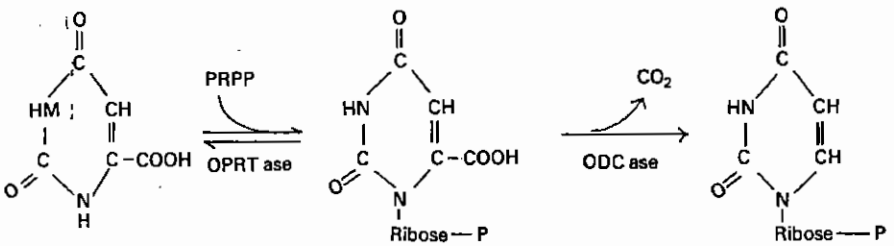
Orotidylate decarboxylase catalyses the irreversible decarboxylation of orotidine 5' -monophosphate (OMP) to uridine 5' -monophosphate (UMP) in *de novo* pyrimidine biosynthesis.

OMP derives from orotic acid, in the presence of 5' -phosphoribosyl pyrophosphate (PRPP) and an enzyme catalysing the reversible reaction, orotate phosphoribosyltransferase (OPRTase). The reaction sequences are shown in FIGURE 1 (Smith, 1973).



OROTIC ACID **OMP** **UMP**

The two enzymes have been co-purified from many tissues, though OPRTase activity is often lost in the process. For example, ODCase has been purified approximately 600-fold from cow brain (Appel, 1968), the enzymes from calf thymus have been separated using starch gel electrophoresis, ODCase activity having been purified 600-fold (Kasbekar *et al.*, 1964) and Brown *et al.* (1975) have purified ODCase from human erythrocyte approximately 300-fold.

In this study we carried out the partial purification of ODCase from human brain using the method described by Appel (1968) for cow brain. Preliminary characterisation has included the determination of the pH optimum, of the K_m and of inhibitors and heat upon enzyme activity.

MATERIALS AND METHODS

MATERIALS

Human brains were collected from Prince of Wales Hospital and were stored at-20°C until used. All compounds used in this experiment were available from Prof. W. J. O'Sullivan's laboratorry, School of Biochemistry, University of New South Wales. The enzyme was assayed by the collection of $^{14}CO_2$ from ^{14}C-OMP which was employed during this study.

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Two different concentrations of ^{14}C-OMP were prepared (36536 dpm/nmole and 21928 dpm/nmole). (The DEAE-cellulose was prepared by suspension in deionized water. It was then made 0.2N with respect to KOH. The DEAE-cellulose was allowed to settle and the fluid was decanted. It was then washed with distilled water on a Buchner funnel until the pH approached 7. It was then suspended in 0.2 N HCl and washed similarly with distilled water until the pH approached 6. It was stored at 4°C in 0.01 M potassium phosphate buffer, pH 7.5, until used.)

METHODS

Enzyme preparation

1. *Initial preparation*

20 gram human brain was homogenized in 20 ml 0.01 M potassium phosphate buffer, pH 7.5, for 1 minute. The homogenate was centrifuged at 10,000 rpm for 30 mins in an SS-34 rotor of Sorvall refrigerated centrifuge. This supernatant was employed for an initial determination of the K_m of the enzyme.

2. *Enzyme purification*

Step 1: Preparation of the homogenate supernatant

507 g human brain was homogenized in a blender at high speed in 625 ml 0.01 M potassium phosphate buffer, pH 7.5 for 1 min. in the cold room. The homogenate was centrifuged at 10,000 rpm for 30 minutes in a GSA rotor of Sorvall refrigerated centrifuge. The volume obtained was 645 ml.

Step 2: pH adjusment

Using 50% acetic acid, the supernatant was adjusted to pH 5.2, followed by centrifugation at 10.000 rpm for 30 minutes in a GSA rotor. The supernatant obtained was 620 ml.

Step 3: DEAE cellulose fraction

The supernatant was adjusted to pH 7.0 and then stirred with 200 g of washed DEAE cellulose for 30 minutes and filtered on a Buchner funnel. The enzyme was absorbed by DEAE-cellulose. The DEAE-cellulose-enzyme was washed with 0.01 M potassium phosphate buffer, pH 7.5. The enzyme was eluted by suspending DEAE-cellulose enzyme in 600 ml of 0,5 M potassium phosphate buffer, pH 7.5, by stirring it for 30 minutes. The mixture was then filtered on a Buchner funnel and washed with the addition of 0.5 M potassium phosphate buffer for 30 minutes. The volume of solution obtained was 895 ml.

Step 4: Ammonium sulphate fractionation

The filtrate was heated for 30 minutes at 60°C and brought to 40% saturation by the addition of 218.74 g ammonium sulphate. Following centrifugation at 10,000 rpm for 15 minutes in a GSA rotor, the supernatant was brought to 60% saturation by the addition of 128 g ammonium sulphate. The precipitate was taken up in 12 ml of 0.01 M potassium phosphate buffer and was then used for enzyme assays.

Enzyme assays

The assay of ODCase was performed by incubating 25 umoles of potassium phosphate buffer, pH 7.5, varying concentrations of ^{14}C-OMP and varying concentrations of the enzyme in a total volume of 1.0 ml. The reaction was shaken at 37°C for 60 minutes in a water bath in a tightly stoppered 20 ml flask with double glass centre wells containing 100 ul of hydroxide of hyamine and was terminated by the injection of 0.2 - 0.5 ml of 0.6 M perchloric acid through the rubber caps. The flasks were shaken an additional 15 minutes to trap all the $^{14}CO_2$ evolved (Fox *et al.*, 1971).

The glass centre wells were removed from the flasks and suspended in a vial with 10 m. of scintillating mixture containing 5% W/V 2,5-diphenyloxazole (PPO) in toluene. The samples were counted in a Packard Tricarb Scintillation spectrometer 2650.

Protein was determined by the microbiuret method.

RESULT

Enzyme preparation

1. *Initial preparation*

Determination of K_m using the supernatant from the initial preparation was performed over a large range of substrate concentrations with 200 ul of enzyme solution (4.2 mg protein enzyme).

The K_m values were obtained, *i.e.* 3.3 uM and 1.66 uM respectively (Fig. 2). The greater value seems to be similar to the K_m of the dimer form enzyme revealed by Brown *et al.*, (1975) from human erythrocytes and the K_m of the same enzyme from cow brain (Appel, 1968).

2. *Enzyme purification*

Enzyme purificaion was carried out by heat treatment, ammonium sulphate step without heating the supernatant at pH 5.2 (Appel, 1968).

From the total protein obtained in the last step, ODCase was purified approximately 18.5-fold, as seen in TABLE 1, and yielded 5.4%.

TABLE 1. – Purification of ODCase (Step are as outlined in text).

	Steps	Volume	Protein	Total Protein	Enzyme Activity	Specific Activity	Yield	Purification
		ml	mg/ml	mg		nmoles/hr mg protein	%	fold
I	Homogenate supernatant	645	19.0	12.300	3700	0.30	100.	1
II	pH supernatant	620	14.5	9.000	2900	0.32	73.36	1.07
III	DEAE-cellulose	895	5.5	4.900	2000	0.41	40.17	1.36
IV	Heat/ammonium sulphate	12	55.5	666	1690	2.55	5.43	8.5

The enzyme obtained from the final step of purification was employed for the following studies: the effect of pH, the effect of thiol compounds, the effect of nucleotides and the effect of heat.

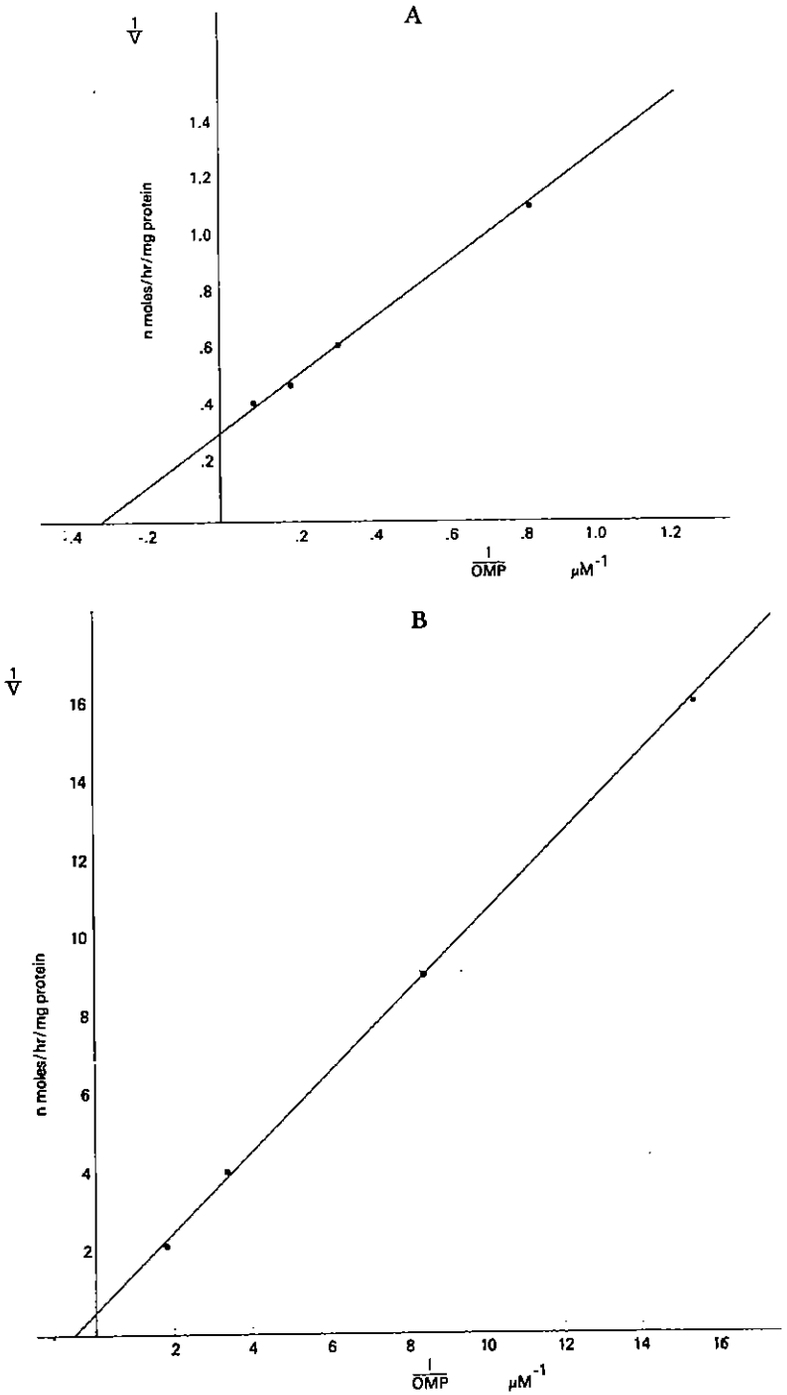


FIGURE 2. — Double reciprocal plots for orotidylate decarboxylase from human brain. A, indicates higher K_m value; B, indicates K_m value.

Effect of pH

The pH activity curve was determined with citrate-phosphate-tris buffer, over a pH range of 5.0 - 6.5 and 5.5 - 8.5 respectively, in two separate sets of experiments. The pattern obtained showed a broad maximum of pH, which is similar to the result of yeast OMP decarboxylase (Creasy *et al.*, 1961) but is different to that of human liver ODCase, which has optimum pH 6.7 at 37°C (Campbell *et al.*, 1977) and rat liver, pH 7.5 (Creasy *et al.*, 1961) and calf thymus ODCase which has optimum pH 7.9 (Kasbekar *et al.*, 1964) (Fig. 3).

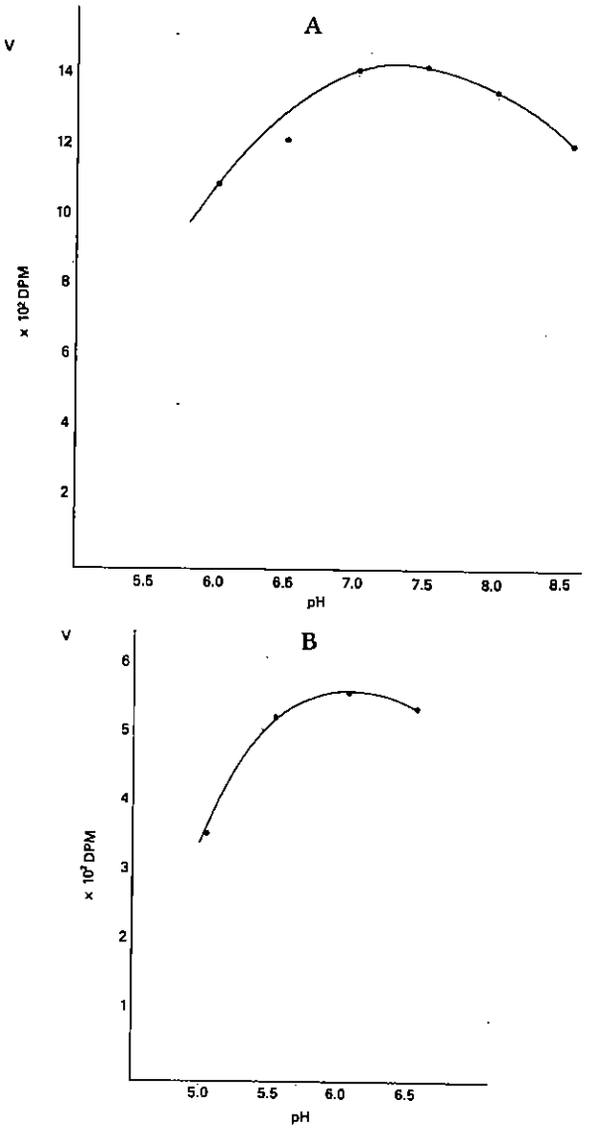


FIGURE 3. — Effect of pH on the activity of orotidylate decarboxylase from human brain. A, indicates higher K_m value; B, indicates lower K_m value.

Effect of thiol compounds

Determination of the effect of thiol compounds upon the enzyme was carried out using B-mercaptoethanol, dithiothreitol (DTT) and reduced glutathione (GSH) with the concentration of 1 mM in assay mixtures. Neither a positive nor a negative effect was observed.

Effect of nucleotides

Twelve compounds were tested as inhibitors of the enzyme. From the result shown in TABLE 2, it is obvious that 6-AzaUMP is the strongest inhibitor for ODCase. UMP and GMP also showed a strong inhibitory effect.

TABLE 2. — Inhibition of orotidylate decarboxylase activity.

	Nucleotides/Nucleotides Derivatives	Relative Specific Activity (%)
	Non	100
	CMP	88.6
+	UMP	42.4
	IMP	99.2
	AMP	96.6
	XMP	9.9
	GMP	68
	6-AzaUMP	0.72
	6-AzaURIDINE	82.4
	6-AzaURACIL	95.4
	Allopurinol	84.7
	CDP	99.2
	Allopurinol (& PRPP)	87.4

All nucleotides were present at final concentration of 1 mM except 6-AzaUMP which was present at final concentration of 0.1 mM.

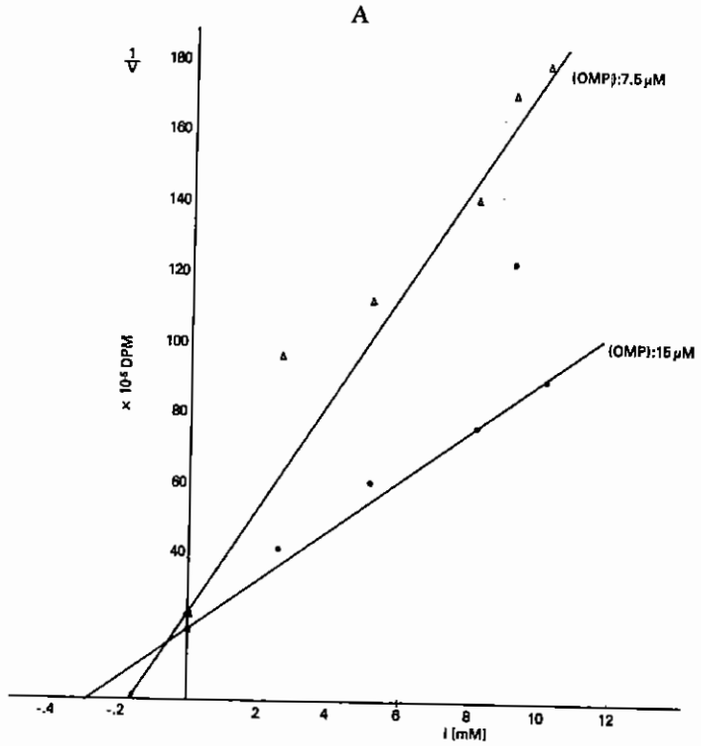
The determination of K_i values of some inhibitors was carried out with two different constant concentrations of OMP (7.5 uM and 15 uM) and various inhibitor concentrations. For 6-AzaUMP, the concentration varies from 0 to 0.06 mM, XMP from 0 to 1.0 mM and GMP from 0 - 1.25 mM (FIG. 4).

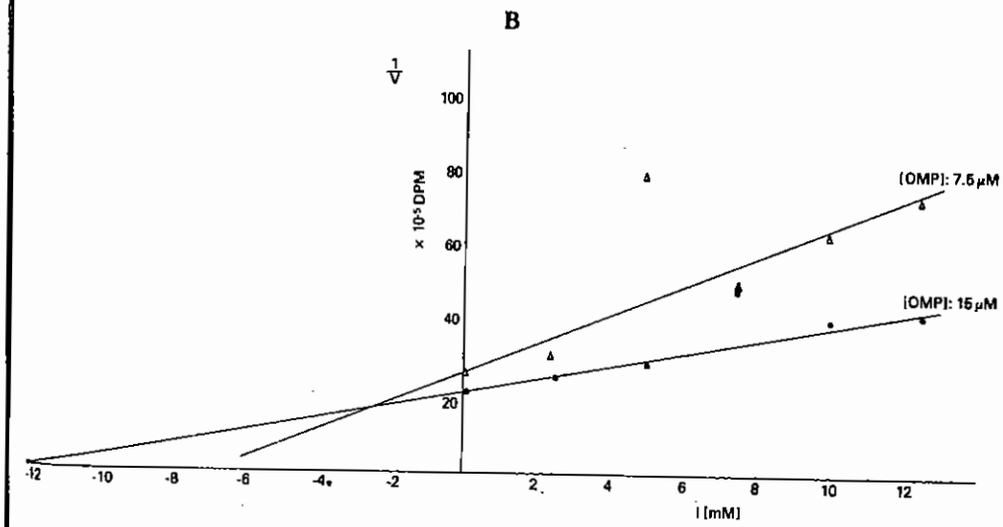
K_i values obtained are shown in TABLE 3.

TABLE 3. — Nucleotide inhibition

	uM
K_m OMP	3.3 1.6
K_i 6-AzaUMP	.25
XMP	60
GMP	180

All K_i values were determined by reciprocal plots of the substrate-velocity curves at several inhibitor concentrations, and assuming simple competitive inhibition.





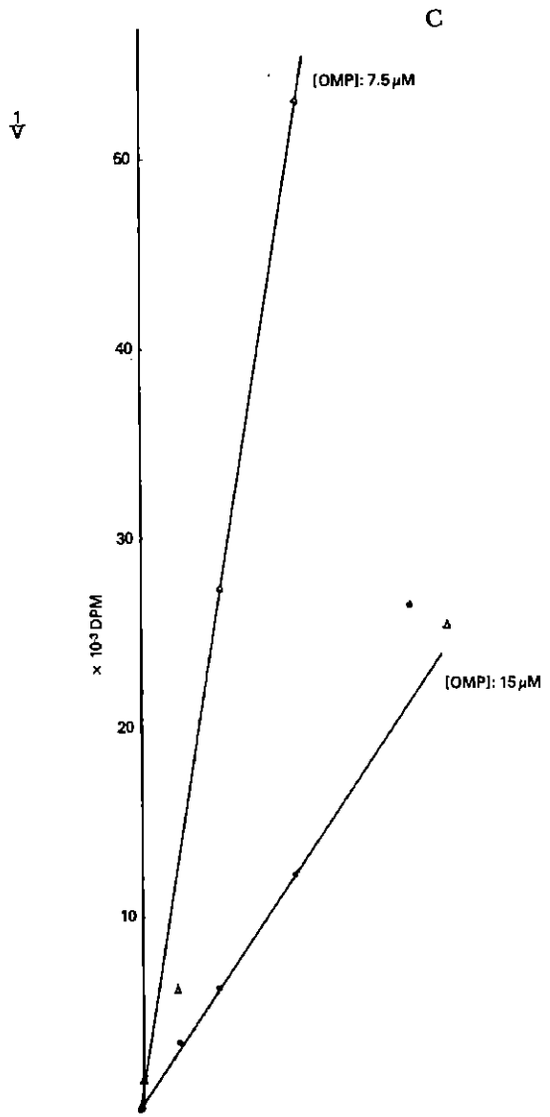


Figure 4. — Kenetics of nucleotide inhibition of orotidylate decarboxylase from human brain. A, varying concentrations of XMP; B, varying concentrations of GMP; C, varying concentrations of 6-AzaUMP.

For 6-AzaUMP, the K_i value was somewhat close to the K_i value of 6-AzaUMP for liver ODCase (K_i = 0.1 uM) (Creasy *et al.*, 1961).

Effect of heat

The effect of heat was carried out using a diluted enzyme solution which had been treated with no inhibitor, 0.1 mM 6-AzaUMP and 1.0 mM XMP.

The heating time intervals were 0; 1; 5; 10; 15 and 20 minutes at 55°C. No obvious trend was observed, though it has been revealed that decreases in enzyme activity were due to incubation and dilution of the enzyme concentration (Fyfe *et al.*, 1973).

DISCUSSION

The conversion of OMP to UMP catalysed by ODCase was carried out by enzyme extracted from human brain.

In the *de novo* pyrimidine biosynthesis, OMP derives from orotate irreversibly by OPRTase. As has been described elsewhere, the activity of the two enzymes remain linked during many of the purification procedures (Appel, 1968; Kasbekar *et al.*, 1964), though Kasbekar *et al.* (1964) have succeeded to separate calf thymus OPRTase from ODCase activity by using starch gel electrophoresis.

Recent study by Appel (1968) revealed that these two enzymes can be co-purified. At least two enzyme in *de novo* pyrimidine biosynthesis have been purified from brain tissue, since in the previous study, brain slices failed to convert orotic acid to UMP.

It appears that the presence of two K_m values represent two oligomeric forms of the enzyme (3.3 uM and 1.6 uM). Brown *et al.* (1975) obtained three K_m values from partially purified human erythrocyte ODCase. The values were 25, 3 and 0,6 uM, which were observed for three oligomeric forms of the enzyme, the monomer, dimer and tetramer with molecular weights of 62.000; 115.000 and 250.000 respectively. The first K_m value (3.3 uM) is also almost similar to that of rat liver ODCase (4.5 uM) (Creasy *et al.*, 1961) and cow brain ODCase (3 uM) (Appel, 1968).

Using citrate-phosphate-tris buffer, over a pH range of 5 - 8.5, indicated a broad maximum pH instead of a sharp peak optimum pH. It is similar to that shown by cow brain ODCase (Appel, 1968) or yeast ODCase (Creasy *et al.*, 1961).

Studies on the activity of ODCase from rat brain indicated that cerebellum has the highest enzyme activity compared with other areas in the brain (Appel, 1968). It is probably related to the high neuronal population and concentration of RNA and DNA in that area. The higher enzyme activity has also been shown in the hemolysate of leukaemia and lymphoma patients (Foster *et al.*, 1973).

In this study partial purification did not take into account which area of the brain was under consideration, because the purpose of this study was not to compare enzyme activity from different areas.

Kinetic studies of the enzyme support other previous studies on ODCase from different sources. Inhibition studies of ODCase by nucleotide derivatives show that 6-AzaUMP, UMP, XMP also GMP inhibit ODCase activity. We found that XMP was also a good inhibitor for human brain ODCase (K_i, 60 uM), whereas GMP was less strong as an inhibitor (K_i = 180 uM). From the effect of these nucleotide derivatives upon the activity of the enzyme, we found that ODCase was inhibited by 6-AzaUMP > XMP > UMP > GMP.

It has been suggested that the inhibitory effect of UMP may be explained by product affinity for the enzyme (Creasy *et al.*, 1961). Studies on liver enzyme results in K_i for UMP 1.5×10^{-4} M, but CMP only inhibited at much higher concentrations, whereas AMP and GMP were inactive. Studies on yeast enzyme resulted in K_i values of 400 uM for UMP, 700 uM for CMP, 800 uM for AMP and 1.6 mM for GMP (Creasy *et al.*, 1961). Blair and Potter (1961) have shown that UMP was a competitive inhibitor of both rat liver and yeast ODCase, whereas uridine and UTP did not inhibit the enzyme. The K_i values were 6.9 mM for the liver enzyme and 2.08 mM for the yeast enzyme.

Studies on heat stability showed that on heating at 55°C the enzyme activity decayed in an exponential manner until approximately 5% remained. Thereafter the slope changed and it decayed at a slower rate (Appel, 1968).

An addition of inhibitors shows the stabilizing effect on the enzyme under conditions of heat. However, it should be noted that decreases in enzyme activity are not merely due to temperature changes (temperature-dependent) but are also due to dilution of the enzyme (Fyfe *et al.*, 1973) which was observed in this experiment.

SUMMARY

Orotidylate decarboxylase has been partially purified approximately 8.5-fold from human brain and some properties of the enzyme have been studied.

Two K_m values were obtained, i.e. 3.3 uM and 1.6 uM, which were thought to be related to two oligomeric forms of the enzyme.

The enzyme was inhibited by 6-AzaUMP > XMP > UMP > GMP, whereas other nucleotide derivatives have only a slightly inhibitory effect.

RINGKASAN

Orotidylate decarboxylase dari otak manusia telah dimurnikan 8,5 kali dan diselidiki beberapa sifat-sifatnya.

Diperoleh dua harga K_m, yaitu 3,3 uM dan 1,6 uM, yang nampaknya berkaitan dengan bentuk oligomerik enzima tersebut.

Enzima tersebut dihambat oleh 6-AzaUMP > XMP > UMP > GMP, sedang derivat-derivat nukleotida yang lain hanya menunjukkan pengaruh hambatan yang lemah.

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