EXPERIMENTAL

A. MATERIALS

Biochemicals and reagents:

Triton X-100, Oxaloacetate, NADH, α-Ketoglutarate, Malate dehydrogenase, Aspartate, Coomassie brilliant blue, Bovine serum albumin, Tris (Hydroxy amino methane), EDTA, Mercaptoethanol, ADP, Malate, Glutamate, Hydrocortisone, Triodothyronine, Dibutyrylated-cyclic AMP were purchased from Sigma Chemical Company, USA.

Chromatography media and reagent for gel electrophoresis:

CM-Cellulose ion exchanger, Acrylamide, N’N’ methylene bis acrylamide, NNN’N’ Tetramethylene diamine, Ammonium persulfate, Bromophenol blue, Coomassie brilliant blue R-250, Glycine, O-Dianisidine tetrazotized salt were also purchased from Sigma Chemical Company, USA.

Other reagents and chemicals:

NaCl, Sucrose, K_2HPO_4, KH_2PO_4, Phosphoric acid, HCl, Mannitol, MgCl_2, KCl, Diethyl ether, Ethanol, Urea, (NH_4)_2SO_4, NaOH, Sodium acetate, Acetic acid, Glycerol, Methanol and all other chemicals were of analytical grade purchased from Sisco Res Lab, Hi-media and Qualigens, India.

Animals: Male Swiss Albino mice (Balb/c strain) were used for the experiments. They were maintained under normal laboratory conditions at 24 ± 2° C and fed with standard pellet diet (Amrut Laboratory, Pune) and tap water ad libitum. Male mice of three age groups (15-, 30- and 60- day) were used for developmental and hormonal studies. Male mice of age groups (15- and 180- day) were used for purification and kinetic studies. Each set of data was collected from 3-4 mice of the same age group. All the operations were performed at a fixed time of the day, in order to avoid fluctuations in enzyme levels due to circadian rhythm and the mice were sacrificed at 1800 hr.

INSTRUMENTATIONS:

pH measurement — A control dynamics digital pH meter model Apx 175 E/C was
routinely used for all pH measurements at room temperature and the calibration was done using standard buffer tablets of different pHs.

Absorbance measurements — A Hitachi U-2000 double beam spectrophotometer was used for all absorbance measurements in the visible and ultraviolet region using glass and quartz cuvettes of 1 cm path length, respectively.

Centrifugation — All centrifugations were carried out in a Hitachi model Himac CR 20B2 - high speed refrigerated centrifuge at 4°C.

Homogenization — Remi motor type RQ - 127A, HP8, rpm 8000 (1.1 Amps, 220/230 V) homogenizer fitted with a teflon pestle was routinely used. Glass homogenizing tubes (5-50 ml) were used for homogenizing tissues.

Electrophoresis — A Bio-Rod slab gel electrophoresis apparatus (Model 122/2.0) and mini protean II chamber was used for polyacrylamide gel electrophoresis.

Weighing Balance — Sartorius balance model 2434 (0.01 mg - 160 g), model 2405 (0.001 mg - 30 g) and an electronic tap pan balance (0.01 - 600 g) were used for weighing chemicals.

B. METHODS

Preparation of homogenates — The mice of various ages were killed by cervical dislocation. The liver and kidney were removed immediately and washed in ice-cold 0.9% NaCl. The adhering blood vessels were blotted dry on a filter paper and the tissues were stored at -70°C till further use. A 10% (w/v) homogenate of the tissues was prepared in ice-cold 0.25 M sucrose using a homogenizer fitted with a teflon pestle.

Fractionation of isoenzymes — The homogenates were centrifuged for 10 min at 800 xg at 2°C to sediment nuclei and cell debris. The resulting supernatant was further centrifuged for 40 min at 14,000 xg at 2°C to sediment mitochondria. The supernatant thus obtained was used for the assay of cytosolic aspartate aminotransferase (c-AsAT) and malate dehydrogenase (c-MDH). The mitochondrial pellet was washed once and suspended in a solubilizing medium (10 mM potassium phosphate buffer, pH 7.5 / 0.25 M sucrose / 0.5% Triton X-100) to make a
10% suspension. Various concentrations of phosphate buffer and Triton X-100 were used to solubilize the mitochondrial AsAT before using the most effective one mentioned above. For the assay of m-MDH, the mitochondrial pellets were suspended in 50 mM potassium phosphate buffer, pH 7.5 containing 0.25 M sucrose. Since it has been observed that the m-MDH is released maximally in this medium. Assays for m-AsAT and m-MDH were performed within 3 hr of mitochondrial suspension.

**Assay of aspartate aminotransferase (AsAT)** — The activity of both the isoenzymes of AsAT (c- and m- AsAT) was measured according to the method of Karmen (1955) with certain modification in the concentration of the substrates and cofactor (Hertzfeld, 1972; Sharma and Patnaik, 1982). According to the method, the rate of oxaloacetate formation is measured spectrophotometrically in a coupled reaction, catalyzed by malate dehydrogenase in the presence of NADH. The reaction occurs as follows:

\[
\text{AsAT} \quad \overset{\text{Asp} + \alpha\text{-KG}}{\xrightarrow{\text{Oxaloacetate} + \text{Glutamate}}} \quad \overset{\text{MDH}}{\xrightarrow{\text{Oxaloacetate} + \text{NADH} + \text{H}^+}} \quad \overset{\text{Malate} + \text{NAD}^+}{\text{Malate dehydrogenase}}
\]

For each mole of oxaloacetate formed from aspartic acid, 1.0 mole of NADH is oxidized in the coupled reaction. NADH has a characteristic absorption maxima at 340 nm with extinction coefficient of 6.22 X 10^6/mole. The rate of disappearance of NADH was measured at 340 nm using a cuvette of 1.0 cm light path. The final volume of the reaction mixture was 3.0 ml which contained:

- Potassium phosphate buffer, pH 7.5 — 100 mM
- α-Ketoglutarate — 6.00 mM
- Malate dehydrogenase — 10 units
- NADH — 0.14 mM
- Enzyme (suitably diluted) — 20 μl
- Aspartate — 75 mM
The reaction was initiated by the addition of aspartic acid to the experimental cuvette and decrease in absorbance was recorded at 30 sec. intervals for 3 min. The linear decrease in absorbance per min was used for the calculation of the enzyme activity. The enzyme was so diluted that the decrease in absorbance did not exceed 0.1 per min.

One unit of enzyme activity was taken as that amount which catalyzed the conversion of one μmole of the substrate per min at 25° C. The specific activity was expressed as units per mg protein.

**Assay of malate dehydrogenase (MDH)** — Both the isoenzymes of MDH (c- and m-MDH) were assayed spectrophotometrically according to the method of Kitto (1969). The method is based on the measurement of the rate of oxidation of NADH (i.e. decrease in absorbance at 340 nm) in the presence of this enzyme and its substrate (oxaloacetate). The reaction occurs as follows:

\[
\text{MDH} \quad \text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow \text{Malate} + \text{NAD}^+
\]

The rate of disappearance of NADH was measured at 340nm in the spectrophotometer using a cuvette of 1.0 cm light path. The final volume of the reaction mixture was 3.0 ml which contained:

- Potassium phosphate buffer, pH 7.5 — 100 mM
- Oxaloacetate — 0.5 mM
- NADH — 0.14 mM
- Enzyme (suitably diluted) — 20 μl

The reaction was initiated by the addition of oxaloacetate. The decrease in absorbance was recorded at 30 sec. intervals for 3 min and the enzyme activity was calculated from the initial rate of oxidation of NADH. The amount of enzyme used was adjusted such that the decrease in the absorbance was met 0.10 per min. One unit of MDH (both in isoenzyme) activity was defined as the amount which is required to oxidize 1 μmole of NADH per min at 25 °C. The specific activity was expressed as units per mg protein.
**Protein estimation** — Protein concentration was determined by the dye binding method of Bradford (1976) using bovine serum albumin as reference standard. The working reagents were as follows:

A. Coomassie brilliant blue G-250 (0.2% in 95% ethanol)

B. Phosphoric acid (85%)

Bradford stock solution was prepared by mixing reagents A and B and was stored in a brown bottle at 4°C for future use. Bradford working solution at the time of use was prepared diluting 15 ml of the Bradford stock solution to 100 ml with distilled water and filtered through Whatman No.1 filter paper. The final concentrations of the reagents were 0.01% (w/v) coomassie brilliant blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid.

 Appropriately diluted protein samples were made upto 1.0 ml with distilled water to which 5 ml of Bradford working solution was added and mixed thoroughly with the help of cyclomixer. Colour was allowed to develop (10-15 min) at room temperature and the intensity of the colour was measured at 595 nm against a reagent blank. Protein concentration was determined with the help of a computed standard curve prepared by taking BSA in the concentration range of 10 to 100 μg.

**Reconstitution of malate-aspartate shuttle** — For the reconstitution of the malate-aspartate shuttle, the tissues were homogenized in four volumes of homogenization buffer (50 mM Tris-HCl, pH 7.4/ 0.25 M mannitol / 1 mM EDTA/ 2 mM MgCl₂/ 30 mM 2-mercaptoethanol) and centrifuged at 800 xg for 10 min at 2°C to sediment nuclei. The supernatant was further centrifuged at 14,000 xg for 30 min to sediment mitochondria. The mitochondrial pellet was washed twice, suspended in homogenization buffer, and used for the reconstitution assay. The post-mitochondrial supernatant was dialyzed for 18 hr at 4°C against 50 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 2 mM MgCl₂, and 30 mM 2-mercaptoethanol. The dialyzed cytosol was centrifuged at 14,000 xg for 30 min to remove traces of particulate materials and the resultant clear supernatant was used for the reconstitution studies.

Reconstitution assay was done according to the method of Cederbaum (1973) and Dawson (1982) with some modifications of our own in the amount of protein taken and in the final assay.
volume. The reaction mixture (final volume, 2.5ml) contained buffer incubation medium (300 mM mannitol/10 mM Potassiumphosphate buffer, pH 7.4/10 mM Tris HCl pH 7.4/10 mM KCl/5 mM MgCl₂/2 mM ADP/2 mM aspartate), 2 mg cytosolic protein and 1 mg mitochondrial protein. After setting the baseline to zero, 50 μl of 7 mM NADH was added to the sample cuvette giving the absorbance of 0.70. The slow steady fall in absorbance was monitored for 2 min and then 50 μl of a solution of 0.2 M each of L-malate and glutamate was added to both cuvettes. The decrease in absorbance was followed upto 10 min.

**Hormone treatments** — Effects of hydrocortisone, triiodothyronine and dibutyrylated cAMP on the activity of AsAT and MDH were studied in the liver and kidney of 15-, 30- and 60-day old male mice. Different doses and time durations have been used to check the optimal dose and time response. Animals were grouped in two sets comprising of 3-4 mice per set. The first set of mice were administered intraperitoneally with hormone suspending medium and served as the normal. The second set of animals were administered with suspended test hormones. To avoid the fluctuation which may arise due to circadian variations, hormone administrations were done at a fixed time of day (0800 hr) in all cases.

**Single hormone injection** — When none of the different doses (0.1, 1.0, 2.5 and 5 mg/100 g body weight) of hydrocortisone administered as a single dose were effective on the shuttle enzymes of liver and kidney, some doses were checked as a repeated dose (for three days). Finally, hydrocortisone (1 mg/100 g body weight) was administered in a total volume of 0.3 ml normal saline containing 10% ethanol. Hormone was administered three consecutive days at the same time. Mice were sacrificed 6 hrs after the final injection and tissues (liver and kidney) were removed and washed in normal saline, blotted dry and stored at -70°C. These tissues were later processed and assayed for MDH and AsAT activities as described under method.

**Dibutyrylated-cyclic AMP (Bt₂ - cAMP)** — Different doses (0.1, 0.2, 0.5 and 1.0 mg/100 g body weight) of Bt₂ - cAMP prepared in normal saline (0.9% NaCl) were administered to the test animals as single dose. All the other conditions followed the similar patterns as described for hydrocortisone.

**Triiodothyronine (T₃)** — Different doses (50, 100 and 200 μg/100 g body weight) of triiodothyronine (T₃) suspended in saline (0.9% NaCl) and 10% ethanol mixture, were adminis-
tered as a single dose. Tissues were similarly removed and stored at -70° C and later used for assay of enzymes.

**Effects of adrenalectomy and hydrocortisone treatments** — Effects of adrenalectomy and the administration of hydrocortisone to adrenalectomised mice were studied on the activities of AsAT and MDH in liver and kidney of mice at three different postnatal ages. The mice of each age group were divided into three sets of 4-5 animals. Set I and II were used for the study of adrenalectomy and adrenalectomy plus hydrocortisone respectively and the III set was used as non-adrenalectomised control.

All the mice of set I & II were bilaterally adrenalectomised. These mice were given in addition to normal pellet diet, 0.9% NaCl instead of water for 5 days following adrenalectomy. During which, endogenous glucocorticoids become negligible in the blood (Raina and Rosen, 1968). On the 6th day, mice of set II were administered intraperitoneally with hydrocortisone (1 mg/100 g body weight) in 0.5 ml of 0.9% NaCl having 10% ethanol. Pilot experiments showed that i.p dose of 1 mg/100 g body weight of hydrocortisone to the mice exhibited maximum effect on the activities of MDH and AsAT. The mice belonging to set I received the same amount of 0.5 ml suspension medium (10% ethanol and 0.9% NaCl) at a fixed time of the day for three days. All the mice were sacrificed after 6 hr of the final hormone injection and their tissues (liver and kidney) were taken out, washed in ice-cold saline, blotted dry and stored at -70° C till the assay of MDH and AsAT.

**Effect of combination of hydrocortisone and Bt_2-cAMP** — Mice of three different postnatal ages were divided into two sets. Following similar pattern as of single hormone administration, combination of Bt_2-cAMP (500 μg/100 g body weight) and hydrocortisone (1 mg/100 g body weight) was administered (i.p) in the test mice of set I in a total volume of 1 ml of 0.9% NaCl/10% ethanol mixture. The second set received only the saline/ethanol mixture. Animals were sacrificed 6 hr after the injection. Tissues were similarly excised, washed in cold normal saline, blotted dry and stored at -70° C until assay of enzyme activities.

**Purification of cytosolic aspartate aminotransferase (c-AsAT)** — c-AsAT was purified from the liver of immature (15 day) and mature (180 day) male mice according to the method of Marra et al. (1977) with some modifications as described earlier (Sharma and Patnaik,
1982). All the steps unless mentioned otherwise, were carried out at 4°C.

Step I — Crude extract

The livers were collected from 10-12 male mice of immature and mature age groups. The adhering blood vessels from the tissue were removed. A 10% (w/v) homogenate of the liver was made in 0.25 M Sucrose using a glass homogenizer fitted with a teflon pestle. The crude homogenate was centrifuged at 22,000 xg for 30 min. The pellet was discarded and the resulting supernatant was used for further purification.

Step II — Ammonium sulfate fractionation

The supernatant obtained from Step I was brought to 40% ammonium sulfate saturation through gradual addition of solid \((\text{NH}_4)_2\text{SO}_4\). The solution was slowly stirred for one hr and was then centrifuged at 10,000 xg for 10 min. The pellet was discarded.

The resulting supernatant of the first \((\text{NH}_4)_2\text{SO}_4\) precipitation was brought to 80% \((\text{NH}_4)_2\text{SO}_4\) saturation through a gradual addition of solid \((\text{NH}_4)_2\text{SO}_4\). After complete solubilization of the added salt into the crude enzyme solution, the pH was maintained at 7.4 by addition of 1 N NaOH. The solution was stirred slowly for 12 hr with the help of a magnetic stirrer. It was centrifuged at 20,000 xg for 30 min and the supernatant was discarded.

Step III — Dialysis

The final precipitate obtained from Step II was dissolved in a minimum volume of 0.02 M sodium acetate buffer, pH 5.4 and was dialyzed for 36 hr against the same acetate buffer. Dialysate was then centrifuged at 10,000 xg for 30 min and pellet was discarded.

Step IV — CM-Cellulose chromatography

The clear supernatant from Step III was applied on to a column (1.6 x 14 cm) of CM-Cellulose which was pre-equilibrated with 0.02 M sodium acetate buffer, pH 5.4 at a flow rate of 30 ml/hr. After application of the sample, the column was extensively washed with 0.02 M acetate buffer pH 5.4. Subsequently, a linear gradient of sodium acetate buffer (0.06-0.16 M) was applied with the help of a gradient mixture to elute the bound fractions. The active peak fractions were collected, pooled and were concentrated by ammonium sulfate fractionation.
Step V — Ammonium Sulfate fractionation

The enzyme-rich fractions obtained after CM-Cellulose chromatography were pooled together and were brought to 80% saturation by the addition of solid (NH₄)₂SO₄. The solution was stirred slowly for 12 hr and then centrifuged at 20,000 xg for 30 min. The supernatant was discarded and the pellet was dissolved in a minimum volume of 0.02 M sodium acetate buffer pH 5.4. This preparation was further dialyzed for 24 hr to remove the salt present with purified enzyme. The sample was then centrifuged at 10,000 xg for 30 min and the clear supernatant was used as the source of purified enzyme for the kinetic and other analysis.

Polyacrylamide gel electrophoresis of purified c-AsAT — Polyacrylamide gel electrophoresis (PAGE) of the purified c-AsAT from the liver of immature and mature mice was performed according to the method of Davis (1969) with slight modification. A 7.5% slab gel was prepared by taking 2.5 ml of acrylamide, 0.133 ml of 1.0 M tris buffer (pH 8.8), 3.73 ml of distilled water, and 0.033 ml of APS (freshly prepared).

Prior to addition of APS, gel solution was degassed for 15 min under vacumm and was immediately casted into slab plates fitted with spacers, sealed at both sides and lower ends. The gel was allowed to polymerize and then pre-run for 15 min at 12 mA. Purified enzyme preparation (50 μg) from both the ages of mice were mixed with assay buffer, glycerol (10%) and bromophenol blue (0.1%). 20-40 μl of this preparation was carefully applied with the help of micro syringe onto gel lanes and electrophoresed for 30-40 min at 24 mA in the cold. Subsequently, one set of gel was removed, washed in distilled water and stained with 0.5% coomassie brilliant blue (prepared in 30% methanol and acetic acid) for 30 min. Background stain was removed by immersing the gel in destaining solution (20% methanol and 7% acetic acid).

The other set of gel, after electrophoresis, were processed for specific staining of c-AsAT according to the method of Doonan (1980) with certain modifications. The staining mixture contained L-aspartic acid (15 mM), 2-oxoglutarate (6.8 mM), tris (100 mM; pH 7.5). Prior to use, O-Dianisidine-tetrazotized (Fast Blue B salt) was added to a final concentration of 10 mM and the staining mixture was stirred vigorously. The mixture was poured onto slab gel and the enzyme activity band appeared as violet colour within a short span of time. The staining mixture was then decanted and the gel were washed thoroughly to avoid any further development of background colour. These gels were stored overnight in a solution of distilled water, methanol
and acetic acid in the ratio of 5:3:1 (v/v) and subsequently photographed.

**Kinetic studies**

*Effect of [L-aspartate] and [α-ketoglutarate] on purified c-AsAT* — The activity of purified c-AsAT from the liver of immature and mature mice was measured at various concentrations of L-aspartate (0-100 mM) and α-ketoglutarate (0.01-10 mM) in separate sets of experiments. The values of $K_m$ for both substrates were determined by Michaelis-Menten and Lineweaver-Burk plots of data using Sigma enzfitter programme (Perella, 1988).

*Effect of [Amino-oxyacetic acid] on c-AsAT* — The effect of amino-oxyacetic acid (AoAA) on the activity of purified c-AsAT from the liver of immature and mature mice was studied by using various concentrations (0-0.2 mM) amino-oxyacetic acid at two different fixed concentrations of L-aspartate (10 and 40 mM) and α-ketoglutarate (1 and 2 mM). The $K_i$ values were determined by Dixon's plot of the data using enzfitter programme.

*Effect of urea on c-AsAT* — Using varying concentrations (0.01-8 M) of urea as denaturant in potassium phosphate buffer, pH 7.5, the activity of purified c-AsAT from two different ages (immature and mature) were studied. The enzymes samples were incubated for 30 min at these concentrations of urea, while the other conditions of assay remained the same. The results similarly expressed as percentage activity retained in presence of specific urea concentration taking no urea as 100%.

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