CHAPTER II

MATERIALS AND METHODS

In this chapter materials and methods used generally throughout the thesis are described.

Materials

A. Animals

Adult male Wistar rats (200-250 g.) were obtained from The National Laboratory Animal Center, Thambon Salaya, Nakornpatom, Thailand. Lab chow (Gold Coin Mills, Singapore) and Bangkok tap water were allowed ad libitum.

B. Chemicals

General reagents were analytical grade supplied by local distributor. Certain substances and chemical were obtained from the following sources :-

Sigma Chemical Co., St. Louis, Mo, U.S.A. :-

Acetylacetone
Ammonium acetate
DL-alanine
L-alanine
Aminopyrine
Barium hydroxide
Bovine serum albumin (fraction V)

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Calcium chloride

2,4-dinitrophenylhydrazine

β-D-fructose

D-Glucose

Glycogen

α-ketoglutaric acid

L (+) lactic acid (Sod. salt)

Magnesium chloride

Magnesium sulphate

Potassium phosphate (monobasic) KH_2PO_4

Potassium chloride

Pyruvic acid (Sod. salt)

Semicarbazide

Sodium chloride

Sodium bicarbonate

Sodium hydroxide

Sodium phosphate (dibasic) Na_2HPO_4

Trichloroacetic acid

BHD Chemical LTd., Poole, England :-

Cadmium chloride

D-Glucose

Thiourea

Trypan blue

E. Merk :

Ethanol absolute

Glacial acetic acid

Perchloric acid

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Phenol

Potassium hydroxide

Sulfuric acid concentrated

O-toluidine

Zinc sulfate

May & Baker Ltd., Dagenham, England :-

Ether anesthetic

NOVO Ind., Copenhagen, Denmark :-

Heparin 5,000 IU/ml

C. Instruments

Dissecting instruments

Disposible tuberculin syringe with needle

Operating table

Metabolic shaker bath (HETOFRIG, HETO, Denmark)

Set of perfusion apparatus (Fig. 1)

Microtubule pump (WATSON-MARLOW 501 S, WATSON-MARLO Ltd., Englnad)

Centrifuge (Roto-Uni II, BHG Co., Germany)

Haemocytometer (Buker Chamber)

Light microscope

Torsion balance (Mettler H 30, Mettler Instrument, Switzerland)

Single pan balance (Sartorius 1213 MP Animal, Gottengen, Germany)

Spectrophotometer

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Vortex mixer (CYCLO-MIXER, Clay Adams, USA.)

Autopipets 50-5,000 µl. (Pipetman, Gilson Medical Electronic, France)

Transmission electron microscope (JEM-200 CX, JEOL)

Water bath

Screw-cap plastic tubes

Glasswares

D. Gas Mixtures

All solution were pregassed with carbogen (95% O_2 : 5% CO_2) and prewarmed to 37°C and kept in these conditions throughout the experiment.

E. Composition of Perfusion and Incubation Medium

The composition of physiological solution used for liver perfusion during the isolation of hepatocytes was the modified formula of Krebs and Henseleit (1932). Sodium gluconate was incorporated to allow the chloride ion to be adjusted to a more physiological level (Bretag, 1969). Calcium was omitted to promote the dissociation of hepatocytes (Berry, 1975). The original formula and the modified formula used are set out below.

Constitutuent	Normal-concentration (mM)	
	Krebs-Henseleit	Modified formula
Na^{+}	143.0	147.7
K^{+}	5.9	3.9
Ca^{+2}	2.45	-
Mg^{+2}	1.18	0.74

Cl^-	128.0	97.4
HCO_3^-	24.9	30.0
Phosphate	1.18	2.5
SO_4^{-2}	1.18	0.74
Gluconate ion	–	21.7

These modifications were based on measured values of the relevant constituents in rat blood drawn from the hepatic portal vein and abdominal aorta (Stacey and Priesl1y, 1976).

Incubation medium was prepared by adding calcium chloride (1.02 mM) and Bovine Serum Albumin, fraction V (1.2%) to this modified Krebs-Hensleit physiological solution.

F. Liver Perfusion Apparatus.

The perfusion apparatus used was shown in Fig. 1

1. The water jacketed coiled tube which maintained the perfusate temperature at 37°C.

2. The polyethylene cannula was connected to the coiled tube (1) with a piece of silicone rubber tubing which long enough to move freely and easily to transfer the cannulated liver from the rat body to the liver funnel.

3. The perfusate reservoir was a 100-ml water jacketed (37°C) chamber with a sintered glass opening gas inlet to aerate the perfusate. The perfusate outlet was connected to the other end of the coiled tube(1) with a silicone rubber tubing. The chamber was fixed with a clamp onto the operating table to give 20 cm. height from the table.

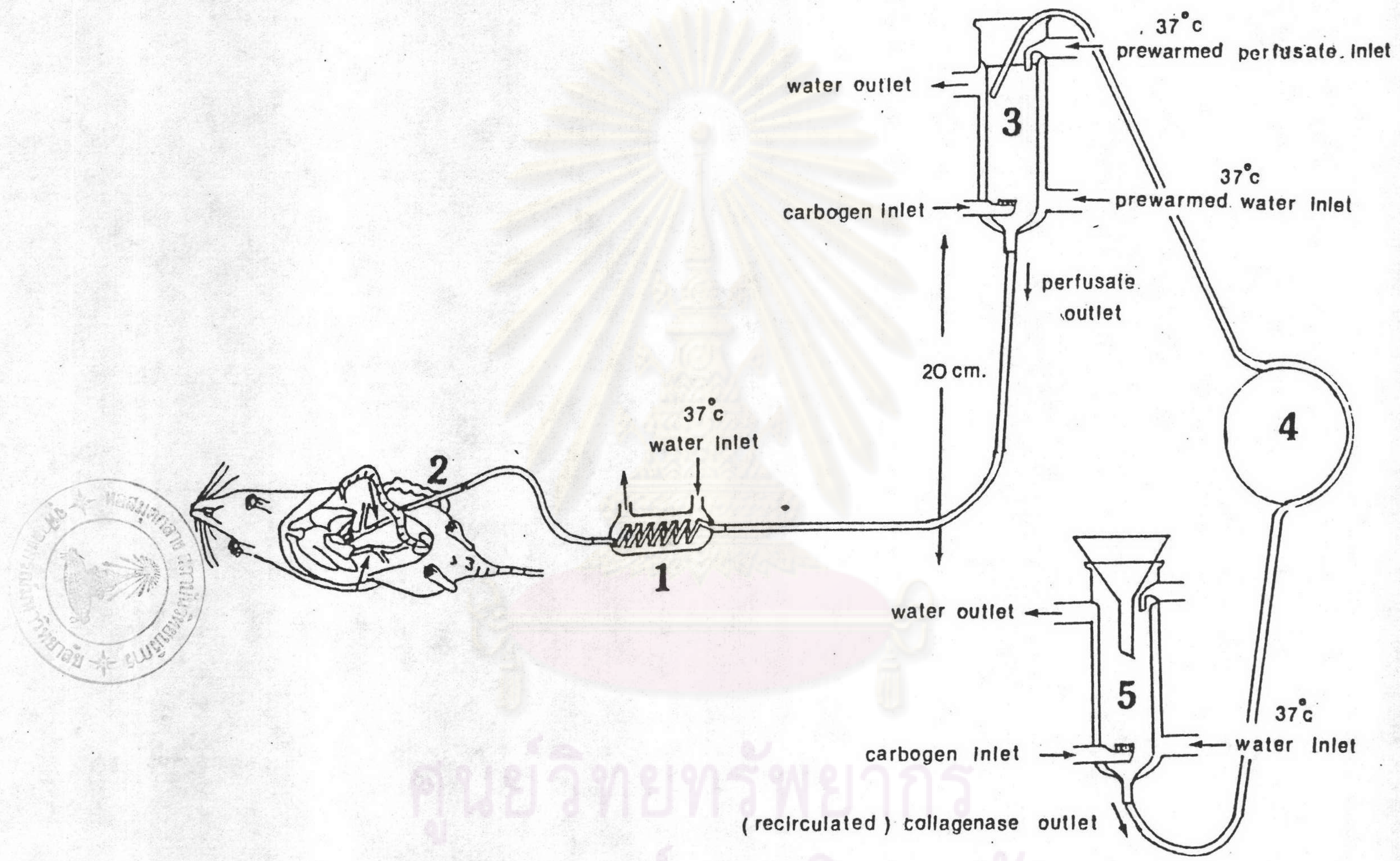

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FIG. 1 LIVER PERFUSION APPARATUS

4. Microtubule pump which delivered perfusate at a rate ranging from 70-100 ml/min.

5. The 100 ml water jacketed ($37^{o}C$) chamber with a liver funnel placed on top. A silicone rubber tubing from this perfusate outlet was connected to chamber (3) via the microtubule pump (4).

6. A 2-litter erlenmeyer flask (not shown) was used as the total buffer reservoir placed in a thermoregulating metabolic shaker bath. A long silicone rubber tubing was inserted into the flask and the other end leading through the pump to the chamber (3). Carbogen was led into the flask through a silicone rubber tubing.

7. A thermoregulating metabolic shaker bath (not shown) which would supply the prewarmed water flowing through the outer jackets of the apparatus (1), (3), (5). This bath was also used for the later incubation of hepatocytes.

Methods

A. Isolated of rat hepatocytes

Hepatocytes were isolated by the method of Berry and Friend (1969) with some modifications by Stacey and Priestly (1978). Surgery was performed at about 9.00 AM for each experiment. The following procedure was used routinely for hepatocyte preparation (Seglen, 1976; Pramyothin, 1980).

Surgical Procedure

The rat was placed in a 5-litter sealed desicator with a big piece of cotton wool soaked with 3 ml of ether. After a few minutes the hypnotized rat was rapidly transfered to the operating table. The

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rat should lie on its back and a very light anesthesia was maintained by placing the opening of a 25 ml-beaker, containing a cotton wool soaked with 1 ml of ether, infront of the rat's nose.

The abdomen was opened by a midventral incision and the liver exposed. Connective tissue surrounding the liver was carefully disjoined. The oesophagus and accompanying blood vessels were doubly ligated and cut between the two ties. Intestines were then removed to the left of the animal to expose the portal vein. The small blood vessel joining the portal vein and running close to the bile duct was ligated. Three loose ligatures were placed around the portal vein and 0.1 ml of heparin (5,000 IU/ml.) was injected into the inferior vena cava, the injection site was pressed with a piece of gauze for a few seconds to prevent bleeding.

The distal ligature was then tied and tension was placed to the vessel via this tie. One of the proximal ligature was lifted slightly to reduce back flow of blood from the liver. With fine scissors a cut was then made in the portal vein, the cannula was immediately inserted, pushed past the proximal ligatures and then tied tightly into place with these two ligatures.

Perfusion Procedure.

When the ligatures were secured, the inferior vena cava and the posterior vena cava were cut, and perfusion was started with Ca^{2+}-free physiological solution at a rate of 70 ml/min. from a 100 ml water jacket (37°C) chamber at a constant pressure of 20 cm of water. The initial perfusion was performed in situ, since in this position there is less danger of flow interruption due to compression, bending,

or twisting of the blood vessels than when the liver was being cut. The washout of blood could be improved by gently moving the liver lobes with fingures. A sign of adequate perfusion seemed to be that the liver cleared immediately and completely. While the perfusion with Ca^{2+}-free buffer was continued, the liver was excised and removed from the carcass and transfered to the funnel placed on top of the second 100 ml water jacketed (37°C) chamber. The perfusion was restarted immediately with recirculating Ca^{2+}-free physiological buffer containing 50 mg% collagenase (Sigma, type IV) the pressure was kept constant at 20 cm water. Perfusion with collogenase buffer was continued for 10-15 minutes, until the liver looked swollen and pale, indicating an adequate digestion of the intracellular matrix.

Purification of the Parenchymal Cells

The liver was then removed and placed in a 100 ml beaker containing 20 ml of 50 mg% collagenase buffer. The capsule was disrupted gently with a spatula and shaken in order to liberate the cells from the connective vascular tissue, and the volume was made up to 50 ml with collagenase buffer from the recirculating medium. The dispersed liver was incubated for 10 minutes on a metabolic shaker bath at 80 oscillation /minute and 37°C in 2 x 250 ml Erlenmeyer flask under an atmosphere of carbogen.

At the end of 10 minutes Bovine Serum Albumin (BSA) was added to give a final concentration of 12 mg/ml and the cell suspension was sieved through nylon meshes to remove connective tissue debris and clumps of infarctious tissue. The resulting initial cell suspension is a mixture of parenchymal and nonparenchymal cells. Hepatocytes

were separated from other cells and cellular debris by differential centrifugation (500 g, ½ min, room temperature) with this fresh medium, 2-3 times, supernatant was discarded each time. Then washed once with incubation medium (modified Krebs Henseleit physiological solution containing 12 mg/ml BSA and 1.62 mM calcium chloride). Finally cells were resuspened in this medium at the concentration of approximately 10×10^6 cells per ml.

Cell viability was routinely estimated by trypan blue exclusion test. Cell preparations with a trypan blue exclusion index of less than 90% were not used for further study.

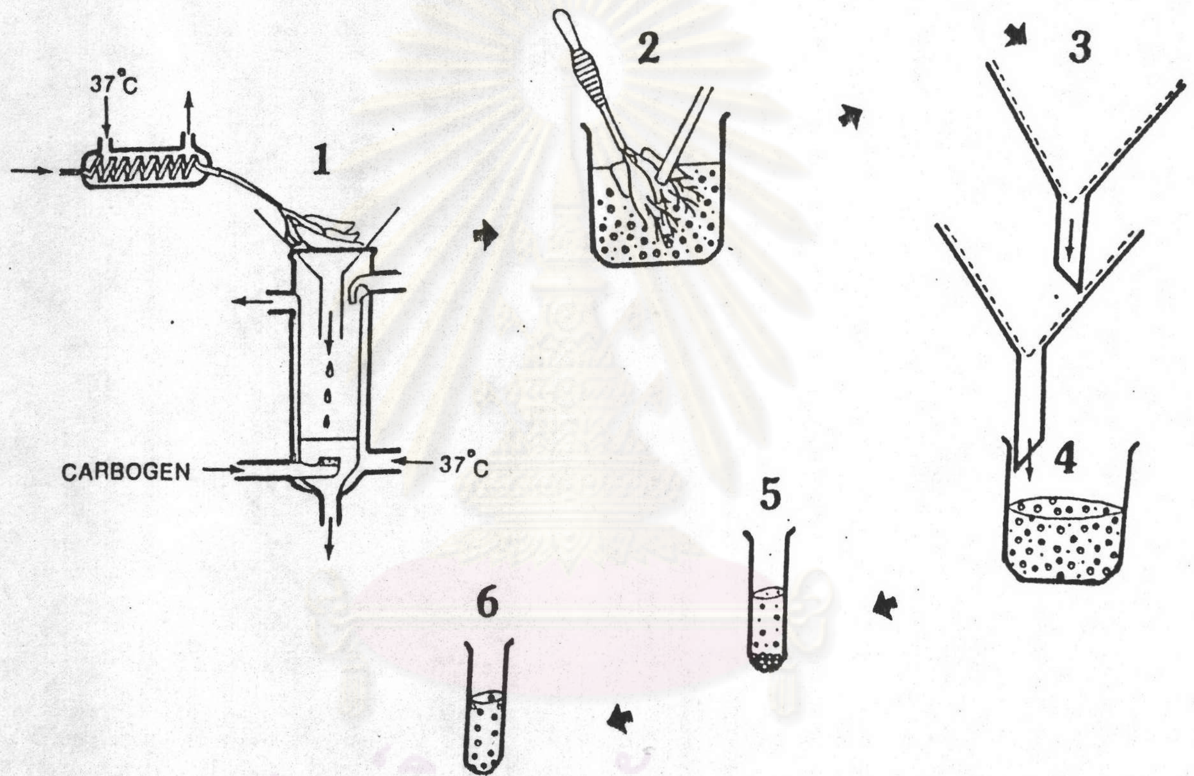

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FIG. 2 PREPARATION AND PURIFICATION OF ISOLATED HEPATOCYTES

Fig 2. Preparation and purification of isolated hepatocytes.

1. Two-steps perfusion of isolated rat liver : first with Ca^{2+}-free buffer (nonrecirculating), then 10-15 minutes with collagenase (recirculating).
2. Mechanical disruption of collagenase-perfused tissue with a blunt spatula. Preincubation of the resulting cell suspension (10 minutes at 37°C in two 250 ml Erlenmeyer flasks under carbogen with shaking in metabolic shaker-bath) may aid the subsequent purification (intact cells round up, damage cells become lighter).
3. Filtration through nylon mesh.
4. The initial cell suspension:a mixture of intact hepatocytes (open circle), damaged hepatocytes (large black dots) and nonparanchymal cells (small black dots).
5. Purification of hepatocytes by differential centrifugation : sedimentation 3 times at 500 g for ½ minute .
6. Final cell suspension of purified and intact hepatocytes.

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B. Incubation of Hepatocyte Suspension

Three replicate hepatocyte suspensions (3.0 ml) were incubated in 25 ml rubber stoppered erlenmeyer flasks. Carbogen (95% O_2 : 5% CO_2) was continuously supplied into each flask via a gas manifold of fine polyethene tubings throughout the incubation period. Incubation were carried out at 37°C and 80 oscillations per minute in a metabolic shaker bath.

C. Determination of the Wet Weight

All experimental result were expressed on a wet weight basis. The wet weight was determined according to the method of Seglen (1976 a). Douplicate samples (3.0 ml) were centrifuged at 3,000 g for 5 minutes, and then inverting the tubes, after 15 minutes, any fluid on the tube walls was wiped off and the tubes were weighed separately. The net weight was obtained by lessening with the individual empty tube weight. the average value was used as the representative wet weight of each experiment.

D. Trypan Blue Exclusion Test

Reagent

Isotonic 0.6% trypan blue solution . Trypan blue 1.50 mg plus 120 mg sodium chloride were disolved in 25.0 ml of distilled water.

Procedure

A 50 μl of an isotonic 0.6% trypan blue solution was mixed with an equal volume of cell suspension. Cell mixture was counted in

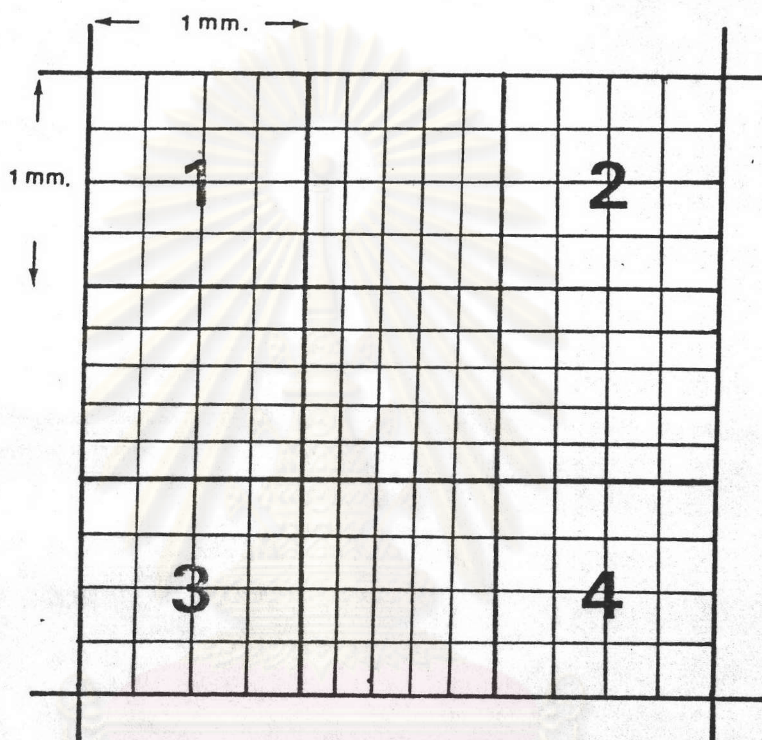

Fig 3. Grid marking on a standard hemocytometer. Cells were counted from the 1st, 2nd, 3rd and 4th square which held the total volume of $4 \times 0.1\ mm^3$ of diluted cell suspention.

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a Burker chamber. The cover glass was mounted. Cell suspension was applied at the edge of the cover glass and was drawn into the chamber by capillary force. Cell counting was performed under the light microscope at 40 x magnification. Cells were counted from the 1 st, 2 nd, 3 rd, and 4 th square (Fig. 3) which held the total volume of 4 x 0.1 mm^3 of diluted cell suspension. In case of hepatic parenchymal cells, intact cells with their yellow color, well defined outline, and refractile appearance were readily distinguished from the flattened, ground-glass looking damaged cells.

The trypan blue exclusion index (% viability) was defined as the number of damaged cells divided by the number of total cells x 100%.

E. <u>Determination of Plasma Enzyme Activity of Alanine Aminotransferase (ALT)</u>

Aliquots (1.0 ml) of cell suspensions were centrifuged at 1,000 g for 1 minute. Cell pellet was analyzed for intracellular K^+ (flame photometry), and the supernatant for ALT.

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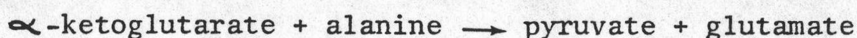
ALT activity was always measured on the day of the experiment using the method of Reitman and Frankel (1957). The reaction are as follow.



<u>Reagent</u>

Phosphate buffer, 0.1 M, pH 7.4. Mixed 420 ml of 0.1 M disodium phosphate and 80 ml of 0.1 M potassium dihydrogen phosphate.

Pyruvate, 2 mM (for standard curve). Dissolved 22.0 mg of sodium pyruvate in 100 ml of phosphate buffer (0.1 M, pH 7.4).

α-ketoglutarate, 2 mM, dl-alanine, 200 mM (for ALT substrate). Placed 29.2 mg of α-ketoglutaric acid and 1.78 g. dl-alanine in a small beaker. Added 1 N sodium hydroxide until the solution was completed. Adjusted to a pH of 7.4 with sodium hydroxide, transfered quantitatively to a 100 ml volumetric flask, and then diluted to 100 ml with buffer solution.

Sodium hydroxide solution, 0.4 N.

2,4-dinitrophenylhydrazine, 1 mM. Dissolved 19.8 mg of 2,4-dinitrophenylhydrazine in 100 ml of 1 N hydrochloric acid.

Procedure

One milliliter of the ALT substrate was pipetted into a test tube, and placed in a water bath at constant temperature ($40^{o}C$) for 10 minutes. After addition of 0.2 ml of sample, the content were mixed and incubated for 30 minutes. When the incubation period was exactly 30 minutes, each test tube was removed from the water bath immediately and 1.0 ml of 2,4-dinitrophenylhydrazine reagent was added to stop the reaction. After standing at room temperature for a minimum of 20 minutes, 10 ml of 0.4 N sodium hydroxide were added, mixed throughly. At the end of exactly 30 minutes, the optical density of the solution was measured at 505 mμ , using water as the blank. Any filter in the range of 490 to 530 mμ may be used satisfactorily.

The standard curve was prepared by mixing 0.1, 0.2, 0.3, 0.4, 0.5 ml of 2 mM pyruvate and 0.9, 0.8, 0.7, 0.6, 0.5 ml of ALT substrate respectively to make the total volume of 1.0 ml. Each concentration was prepared in douplicate and 0.1 ml substrate was used

as blank. After adding of 0.2 ml water, the hydrazine reagent and the alkali were added as described in the procedure. The change in optical density was plotted against the activity of transaminase, expressed in IU as 11.2, 22.5, 37.3, 56.6 and 67.8 respectively.

The activity of alanine aminotransferase of each sample was determined from standard curve and results were expressed as IU per ml.

F. Determination of Intracellular K^+ Concentration

Reagent

Perchloric acid, 3% (w/v)

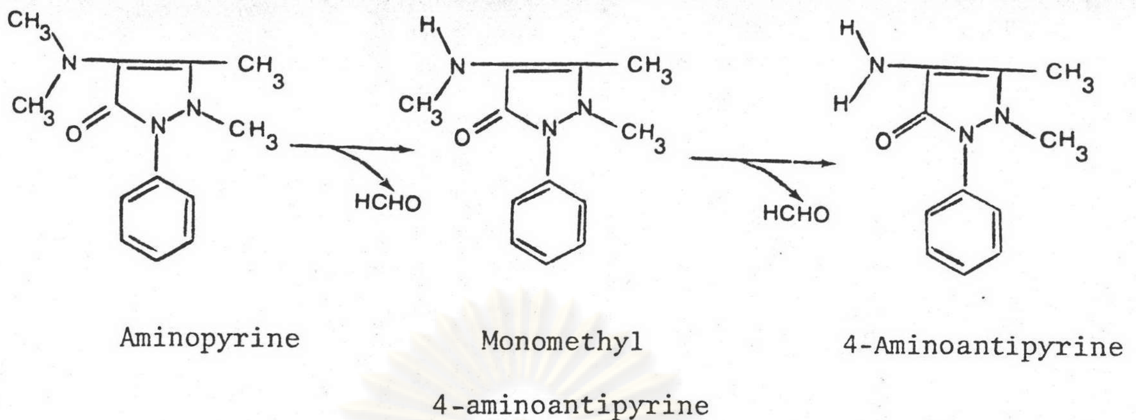
Procedure

Cell pellet (the bottom layer of the centrifuged 1.0 ml cell suspension) was extracted with 2 ml of 3% (w/v) perchloric acid (PCA). After centrifugation at 5,000 g for 5-10 minutes, the resulting clear supernatant was determined for K^+ by flame photometry.

Intracellular potassium content was calculated as μmoles per g cells wet weight.

G. Determination of Microsomal Aminopyrine Demethylase Activity

The microsomal demethylase activity was determined according to the method described by Mazel (1972), using aminopyrine which is a model substrate to study N-dealkylation of drugs. The measurement of the metabolic conversion of aminopyrine is based on the following reaction.



Aminopyrine Monomethyl 4-aminoantipyrine 4-Aminoantipyrine

Formaldehyde formed during the incubation is trapped as semicarbazone and measured by the colorimetric procedure of Nash (1953).

<u>Reagents</u>

Na^+/K^+ phosphate buffer, 0.5 M, pH 7.4

Aminopyrine (5 μmoles), magnesium chloride (25 μ moles), semicarbazide (45 μ moles) in 0.5 M phosphate buffer 0.5 ml. Preweighed and dissolved 238.5 mg of Aminopyrine (97%),101.1 mg of magnesium chloride and 100.4 mg of semicarbazide with 0.5 M phosphate buffer adjusted to 10.0 ml.

Magnesium chloride (25 μmoles), semicarbazide (45 μmoles) in 0.5 M phosphate buffer 0.5 ml (for cell blanks).

Zinc sulfate (15%). Dissolved 15.0 g. of Zinc sulfate in 50.0 ml of distilled water, adjusted to 100.0 ml.

Saturated barium hydroxide. Added barium hydroxide to boiling water until the solution was well saturated. Filtered the hot solution. (noted : crystal growth on cooling)

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Nash reagent. Dissolved 30.0 g ammonium acetate in 50.0 ml of distilled water. Added 0.4 ml acetyl acetone and adjusted to 100.0 ml with distilled water (Kept in refrigerater and discarded if the reagent turned yellow).

Fomaldehyde solution (40% w/v)

Procedure

Triplicate of 3-ml hepatocytes were incubated with 0.5 ml of aminopyrine (5 μ moles) plus magnesium chloride (25 μ moles) and semicarbazide (45 μ moles). Aminopyrine was omitted in the tubes of cell blanks. Incubation was carried out at 37°C with shaking for 30 minutes. At the end of incubation period, flasks were removed from water-bath and the reaction were stopped by adding 2.0 ml zinc sulfate (15%), mixed well and standed at room temperature for 5 minutes. Saturated barium hydroxide (2.0 ml) was added, mixed and leaved at room temperature for further 5 minutes. Then the entire contents were poured into centrifuge tubes and centrifuged for 10 min, using speed high enough to settle all precipitate. Five millilitters of the supernatant were transfered to test tubes. Two millilitters of Nash reagent were added, mixed and placed in water-bath (60°C) for 30 minutes. (Filtration might neccessary if the solutions were cloundy, using Whatman No. 1 filter paper). The absorbance at 415 mμ was measured using cell blank to set the zero absorbance. (Thereby the endogenous formaldehyde formed by the tissue blanks was substracted from the total amount of formaldehyde formed).

A standard formaldehyde curve was prepared by diluting formaldehyde solution (40% w/v) with distilled water to abtain the

following concentrations : 4,2,1 and 0.5 μg/ml. Each concentration was analyzed in douplicate. Aliqouts of 5 ml. of each concentration were pipetted into test tube, and 5.0 ml of distilled water was used as a blank. Two millilitters of Nash reagent were added and mixed well. Tubes were placed in a 60° water bath for 30 minutes and allowed the color to develope. The absorbance of each concentration was measured at 415 mμ . The average optical density of each concentration was plotted versus the amount of formaldehyde present in 5.0 ml pf standard solutions (2.5, 5, 10 and 20 μg, respectively). Quantity of formaldehyde formed from the standard curve was determined and converted to μ moles by deviding with the molecular weight of formaldehyde (30.026). Results were expressed as μ moles formaldehyde formed per g. cell wet weight per 30 minutes.

H. Determination of the Gluconeogenic Capability

The gluconeogenic capability of the isolated hepatocytes was determined using the method of Cornell and Filkins (1974).

Reagents

Phosphate buffer, 0.1 M, pH 7.4.

L-alanine (M.W. 89.09).

Dissoved and adjusted 138.1 mg of l-alanine with phosphate buffer to 5.0 ml.

β-D-fructose (M.W 180.1).

Dissolved and adjusted 279.16 mg of β-D-fructose with phosphate buffer to 5.0 ml.

L(+) lactic acid (M.W. 90.1).

Dissolved and adjusted 139.7 mg of L(+) lactic acid with phosphate buffer to 5.0 ml .

Sodium pyruvate (M.W. 110.0).

Disolved and adjusted 170.5 mg of sod. pyruvate with phosphate buffer to 5.0 ml.

Procedure

Rats were fasted with excess of water for 24 hours prior to the isolation. Aliquots 3.0 ml of hepatocyte suspension were incubated with 0.1 ml of gluconeogenic substrates (L-alanine, β-D-fructose, L(+)-lactic acid, and sodium pyruvate) to give the final concentration of 10 mM, and cell balnks were given 0.1 ml of 0.1 M phosphate buffer. Fasks were gassed with carbogen and incubated at 37°C in metabolic shaker bath (80 oscillations/min.). After 1 hour of incubation, 1 ml-samples were deproteinized with 3% TCA and supernatant of each sample was assayed for glucose using the O-toluidine method.

Gluconeogenic rate was calculated as micromoles of glucose produced per g. of cell wet weight per hour.

I. Determination of Glucose

Glucose was assayed using the O-toluidine method.

Reagents

Trichloroacetic acid (TCA), 3% w/v.

D-Glucose, 25 mg% (for standard curve)

O-toluidine reagent

Dissolved 1.5 g. of thiourea in 940 ml of glacial acetic acid, added 60.0 ml of O-toluidine. The reagent must be kept in refrigerator and protected from light.

Procedure

Aliqouts 1 ml hepatocyte were deproteinized with 3.5 ml of 3% TCA and centrifuged at 1,000 g for 5 minutes to sediment all the precipitate (filtered with Whatman # 1 if necessary). The 0.5 ml of deproteinized supernatant was diluted with equal volume of distilled water to give 1.0 ml sample.

Standards were prepared by diluting the 25 mg% glucose solution with distilled water to obtain the following concentration : 0.5, 0.1, 0.15 and 0.20 mg glucose per 0.6 ml. Water (0.6 ml) was used as blank. The 0.4 ml of 3% TCA was added to each tube to make to final volume of 1.0 ml standards. Each concentration was analyzed in douplicate.

All the samples and standards were added 4.5 ml of O-toluidine reagent and mixed thoroughly. Tubes were placed in boiling water for 8 minutes, then standed at room temperature for cooling. The absorbance at 630 mμ was measured. The average optical density of each concentration of standard glucose was plotted versus the mg glucose presented in 1.0 ml standards. The mg of glucose in samples was determined from standard curve and converted to μ moles by dividing with molecular weight of glucose (180.16).

J. Morphological Examination

Cell counting, cell appearance and trypan blue exclusion tests of isolated hepatocytes were performed using a Nikon optical light microscope.

Transmission electon micrographs of isolated hepatocytes were taken at 3,000 x to 50,000 x magnifications. Cells were fixed with 1.2% OsO_4 in 0.1 M Sorenson's phosphate buffer(pH 7.3) for 2 hours. After washing with phosphate buffer, cells were dehydrated with graded ethanol (from 35% up to 100%) then infiltrated with propylene oxide and plastic (Epon-812) for 2-3 days. The embeded cells were sectioned with ultramicrotome and stained with uranyl acetate and lead citrate prior to photomicrography.

K. Expression of Results and Statistics

All experimental results were expressed using the g wet weight of cell as the reference point, and generally expressed as mean ± S.E. of 5 separated experiment. Within each experiment, 3 replicate samples were used to generate mean values.

The statistical significance was determined by the Student's t-test. The level of significance was set at $p < 0.05$.

Linear regression was used to estimate the quantity of each observed value from its standard curve prepared within each experiment.

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