Chapter II

Changes in the glucose, lactic and keto acid contents of the medium during cultivation of normal, neoplastic, newborn and regenerating rat liver

Chapter II

Changes in the glucose, lactic and keto acid contents of the medium during cultivation of normal, neoplastic, newborn and regenerating rat liver

As stated earlier, the aim of these investigations was to get certain comparative biochemical data on normal and neoplastic liver and to study the differences between the two with regard to nutritional and metabolic characteristics. To rule out the possibility that the differences observed are due to those between fast-growing and other tissues, additional studies were also made on two other tissues of the former kind, namely, regenerating and newborn liver.

The tissue culture technique was used for the study of nutritional characteristics as it was found from studies made in this laboratory that explants of rat liver tissue can be maintained in a fully defined synthetic medium in a metabolically active state for a period of nine days although they fail to exhibit growth.

The present chapter describes studies on glucose requirement in relation to lactic and keto acid production in the different tissues studied.

Experimental

Glassware

Standard rimless pyrex test tubes of size 5" x 5/8" were used in these experiments. They were cleaned and sterilized by the procedure described below:

The tubes were left overnight in chromic acid and washed free of acid by running tap water. They were then scrubbed, rinsed with detergent and washed successively with tap water, ordinary distilled water, and glass distilled water. They were then drained, heated in an oven at 140° for 3 hours, stoppered with gauze plugs, wrapped in craft paper, and autoclaved at 20 lb. pressure for 15 minutes. The autoclaved packets were dried at 80°.

A similar procedure was used for cleaning and sterilizing other glassware used in these experiments.

Rubber stoppers were first left in 5% sodium bicarbonate solution at 60° for 30 minutes, scrubbed throughly, washed first under running tap water and then with glass distilled water, dried with a piece of clean washed gauze, and packed in large test tubes treated similarly and stoppered with cotton plugs. The tubes and the stoppers were sterilized by

autoclaving at 15 lb. pressure for 20 minutes amd used as required.

Chemicals

The chemicals used in these experiments were obtained from the following sources:-

Inorganic salts, L-cysteine hydrochloride, L-cystine, DL-methionine, thiamine hydrochloride, riboflavin, vitamin K, calciferol, tocopherol phosphoric acid, calcium pantothenate, biotin, niacin, pyridoxine hydrochloride, cholesterol, casein and anthrone were obtained from E.Merck, Darmstadt, Germany.

Glycine, DL-, alanine, serine, isoleucine, aspartic acid, glutamic acid, valine, phenylalanine, threonine, tryptophane, L-, leucine, proline, arginine hydrochloride, histidine hydrochloride, lysine hydrochloride, tyrosine, phenol red and glucose were obtained from British Drug Houses, Ltd., England.

Vitamin C, folic acid, niacinamide, vitamin B_{12} were obtained from Hoffmann La Roche Co., Basle.

L-glutamine, adenosine triphosphate and monophosphate, ribose, deoxyribose, glutathione, and hypoxanthine were obtained from Sigma Chemical Company, U.S.A.

Inositol and Tween 80 were obtained from Atlas Powder Company, Delaware; and choline chloride, from Chemo Puro manufacturing Corporation, New York.

3'-Methyl-4-Dimethylaminoazobenzene was supplied by Dr.W.T.Burke of West Virginia University School of Medicine, Morgantown, U.S.A.

Subjects

The subjects were adult male albino rats weighing about 150-200 g. at the commencement of the experiment. They were members of a colony developed by inbreeding for successive generations during the past four years. The parent stock had been obtained from Haffkine Institute, Bombay.

The rats were divided into two groups, experimental and control, and kept four in a cage with food and water ad libitum. The composition of their basal diet was as follows:-

Casein	150gm	Choline	500mg
Rice flour	270gm	Thiamine	3mg
Sucrose	540gm	Riboflavin	2mg
Salt mixture USP XIV	40gm	Pyridoxine	2.5mg
Cod liver oil	40c.c.	Calcium pantothenate	7mg

The experimental group received in addition 3'-Methyl-4-Dimethylaminoazobenzene. The dye was dissolved in groundnut oil and incorporated at the level of 600 mg. per kilogram of diet.

Tissues

Tumors could be detected in the dye-fed rats after an experimental treatment of about six months. When the presence of tumours was suspected, the animals were subjected to laprotomy and the tumors dissected out if found to be adequately developed. Simultaneously with every tumour-bearing animal, a control animal was sacrificed.

The animals were killed by decapitation under light ether anesthesia and the livers perfused with isotonic KCl. The tissues were removed under sterile conditions and freed from connective tissues etc.

The tumor tissues were similarly removed and freed from necrotic and hemorrhagic areas to the extent possible. Only hard tumor portions were used for the experiment.

For studies on regenerating liver, 66 per cent of liver tissue was removed from rats of about the same age and weight as the experimental and control animals by the method of Higgins and Anderson (165). 96 hours after this operation

the animals were killed and the livers taken out immediately. The above interval was chosen since it has been reported that a sham operation may also produce changes in enzyme pattern which, however, return to normal within 3 or 4 days.

For studies on newborn rats, the livers were removed immediately after birth.

For studies on fasted animals, animals of about the same age and weight as the controls were fasted for 48 hours during which period they were given water ad libitum. They were sacrificed at the end of this period and their liver tissues removed.

Medium

The synthetic medium used in these investigations was based on that devised by Waymouth (166) with the following modifications:-

1. The concentration of glucose was reduced from 500 mg. to 100 mg. per 100 ml.
2. The level of arginine was raised from 7.5 mg. to 30 mg. per 100 ml.
3. Serine, alanine, calciferol, menadione, tocopherol, ATP, AMP, ribose and deoxyribose were added.

4. Since the 'L' forms of certain aminoacids were not available, the 'DL' forms were used at double the concentrations specified by Waymouth. These amino acids were: tryptophan, methionine, phenylalanine, isoleucine, alanine, serine, valine, aspartic acid and threonine.

The medium was compounded from different stock solutions prepared according to the following compositions and stored at 5 to 10°. It must be pointed out that the solutions 1,2 and 3 were prepared at respectively 2,40, and 10, times the specified concentrations for convenience of storage. However, the final medium was diluted so as to yield the specified concentrations. Glass distilled water was used for preparing the solutions.

Stock solution 1 (S1)

Chemicals	Mg./100 ml.
NaCl	600
KCl	15
$CaCl_2$ $2H_2O$	12
$MgCl_2$ $6H_2O$	24
$MgSO_4$ $7H_2O$	20
Dextrose	100
Ascorbic acid	1.75
Cysteine	9.0
Glutathione	1.5
Choline chloride	25.0
Hypoxanthine	2.5
Glutamine	35.0
Na_2HPO_4	30.0
KH_2PO_4	8.0
$NaHCO_3$	224.0

Stock solution 2 (S2)

Chemicals	Mg./100 ml.
Thiamine hydrochloride	1.0
Calcium pantothenate	0.1
Riboflavin	0.1
Pyridoxine hydrochloride	0.1
Folic acid	0.04
Biotin	0.002
Inositol	0.1
Nicotinamide	0.1
Vitamin B_{12}	0.02

Stock solution 3 (S3)

Chemicals	Mg./100 ml.
L-lysine hydrochloride	24.0
L-Histidine hydrochloride	15.0
L-Glutamic acid	15.0
L-Arginine hydrochloride	30.0
L-Leucine	5.0
L-Proline	5.0
L-Cystine	1.5
L-Tyrosine	4.0
DL-Tryptophane	8.0
DL-Methionine	10.0
DL-Phenylalanine	10.0
DL-Isoleucine	5.0
DL-Alanine	5.0
DL-Serine	5.0
Glycine	5.0
DL-Valine	13.0
DL-Aspartic acid	12.0
DL-Threonine	15.0
NaOH	to pH 7.2

Stock solution 4 (S4)

10 mg. of calciferol were dissolved in 2 ml. of alcoholic cholesterol solution which contained 10 mg. cholesterol per ml. of 95% ethanol. To this were added 10 mg. of vitamin A and 0.1 ml. of menadione solution, the latter at a concentration of 10 mg. per ml. of 95% alcohol. 10 ml. of 5% aqueous tween 80 solution were added to this mixture which was then diluted to 100 ml. with water.

Stock solution 5 (S5)

10 mg. of disodium tocopherol phosphoric acid were dissolved in 100 ml. of water.

Stock solution 6 (S6)

27 mg. of Adenosine triphosphate (disodium salt) were dissolved in 50 ml. of water.

Stock solution 7 (S7)

10 mg. of muscle adenylic acid were dissolved in 100 ml. of water.

Stock solution 8 (S8)

100 mg. of ribose and 100 mg. of deoxyribose were dissolved in 100 ml. of water.

Preparation of the working medium

The composition of the working nutrient medium was as follows:-

S1		50.0 ml.
S2		2.5 ml.
S3		10.0 ml.
S4		0.1 ml.
S5		0.01 ml.
S6		0.2 ml.
S7		0.2 ml.
S8		0.05 ml.
Streptomycin		2.5 mg.
Penicillin		5000 units
Phenol red		1.0 mg.
Water	to	100 ml.

The media were sterilized by passage through sterilized glass filters and stored at 5 to 10°.

Preparation and cultivation of cultures

Unless otherwise stated the operations were carried out in a sterile room maintained at a temperature of 25°.

The tissues were removed under aseptic conditions and transferred to sterilized petri dishes containing Hanks' balanced salt solution (167) to which antibiotics were added. The tissues were washed with two changes of this solution and then chopped and cut into small fragments with the help of curved scissors. The time and mode of mincing were so

adjusted as to obtain fragments of fairly uniform size. The fragments were taken out of the salt solution, washed thrice with the nutrient medium and placed for explantation in the latter. The average wet weights (mg.) per explant of normal, neoplastic, newborn, regenerating, and fasting liver were respectively 2.52, 2.45, 2.50, 2.55, and 2.49.

Each explant was lifted from the petri dish with a stainless steel needle and placed on the inner surface of a sterilized pyrex test tube approximately one centimeter from the bottom of the tube. Four explants were placed in each tube the distance between any two explants being about 4 mm. The tubes were then kept aside for 30 minutes during which period the explants adhered to the glass surface. At the end of this period, 0.5 ml. of the nutrient medium were added to each culture tube, the tubes closed tight with sterilized rubber stoppers and cultivated at 37° by the conventional roller tube method (168) using a roller drum (Cat. no. 2.13, Wyble Engineering Corporation, Silver Spring, Maryland, U.S.A.) rotating at a speed of 12 revolutions per hour. The medium in the culture tubes was renewed at 3-day intervals (72 hours) and the cultures maintained for a total period of 9 days. One hundred culture tubes were set up for each experiment.

Collection of culture fluid

The spent medium was collected in sterilized centrifuge tubes and centrifuged at 1560 x g. for 20 minutes. The resulting supernatant was frozen until analysed. The samples obtained on different days were analysed simultaneously. The incubated medium was used as the control.

Biochemical procedures

Glucose in the medium was determined by the method described by Gothoskar et al. (169), lactic acid, by that of Barker and Summerson (170), and total keto acids, by that of Friedemann et al. (171) Glycogen in the tissues was estimated by the method described by Kemp and Kits Van Heijningen (172).

Results and discussion

The changes observed in the glucose, lactic, and keto acid contents of the medium during cultivation of adult, newborn and regenerating liver tissue explants and of primary hepatoma are shown in Tables 1a to 1d. The data for the 72 hour period are compared in Table 2 and Fig. 1 which also include similar data on fasting liver.

Table 1

Changes in glucose, lactic and keto acid contents of the medium with the progress of cultivation

(Expressed as mg. per 100 ml; + sign denotes increase, and - sign, decrease)

1a. Adult liver tissue

Period of cultivation (days)	Glucose	Lactic acid	Keto acid
3	+ 28.7	+ 2.2	+ 1.5
6	+ 6.6	+ 0.3	+ 0.5
9	+ 1.8	+ 0.1	+ 0.3

1b. Primary hepatoma

Period of cultivation (days)	Glucose	Lactic acid	Keto acid
3	- 20.7	+ 19.1	+ 2.1
6	- 5.4	+ 4.2	+ 0.6
9	- 2.0	+ 1.3	+ 0.4

1c. Newborn liver

Period of cultivation (days)	Glucose	Lactic acid	Keto acid
3	- 79.0	+ 95.0	+ 9.7
6	- 75.8	+ 75.0	+ 5.1
9	- 66.6	+ 32.1	+ 1.8

1d. Regenerating liver

Period of cultivation (days)	Glucose	Lactic acid	Keto acid
3	+ 12.4	+ 3.2	+ 2.2
6	+ 6.8	+ 1.0	+ 1.5
9	+ 3.4	+ 0.7	+ 1.1

Table 2

Comparative data on changes in the glucose, lactic and keto acid contents of the medium during cultivation of normal, neoplastic, newborn, regenarating, and fasting liver tissues

Tissue	Change after 72 hours (mg. per 100 ml. of medium)			Glycogen content of the tissue
	Glucose	Lactic acid	Keto acid	(gm %)
Normal liver	+ 28.7	+ 2.2	+ 1.5	3.5
Neoplastic liver	- 20.5	+19.1	+ 2.1	0.4
Newborn liver	- 79.0	+95.0	+ 9.7	0.9
Regenerating liver	+ 12.5	+ 3.2	+ 2.2	1.6
Fasting liver	+ 5.1	+ 1.2	+ 2.3	0.2

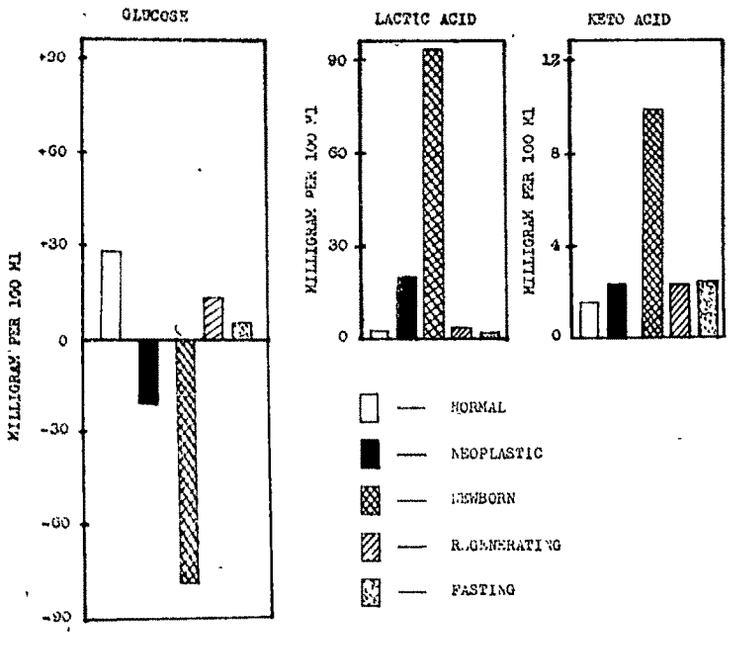

Fig. 1. Changes in the composition of the medium with the progress of cultivation of different liver tissues.

It can be seen from the results that whereas glucose is released into the medium by explants of normal, regenerating and fasting liver, it is utilized from the medium by those of hepatoma and newborn liver. Although the last two resemble each other in that they utilize glucose from the medium, they differ from each other in the rate of utilization, newborn liver tissue showing a greater rate of glucose utilization as well as lactic acid production. The ratio of lactic acid and keto acid produced to glucose utilized is also found to be greater in this tissue. Thus the results point to qualitative differences in glucose utilization between the cultures of primary hepatoma and newborn liver on the one hand and normal and regenerating liver on the other, and to quantitative differences between the former two. The question arises as to whether the similarity of hepatoma to newborn liver is due to its resemblance to fast-growing tissues generally, but this hypothesis derives little support from the observations on regenerating liver. However, it is possible that regenerating liver loses some of its regenerating characteristics when cultivated in synthetic medium.

The differences in the utilization of glucose between the different tissues, and what appear to be parallel

differences in their initial glycogen content, suggest a relation between the two but the data on fasting liver do not fit into this picture. The question therefore arises as to whether the glucose released into the medium is from the glycogen store of the tissue or from de novo synthesis.

The considerable formation of lactic acid in the case of hepatoma and newborn liver is consistent with the high rate of glycolysis generally observed in fast-growing tissues. However, in addition to the quantitative differences pointed out between the two tissues with regard to glucose utilization, there are differences in the ratios of lactic acid and keto acid produced to glucose utilized.

Newborn liver was found to differ from hepatoma as well as other tissues studied in that the cultures showed a considerable amount of proliferation as revealed by microscopic examination.

The higher rate of glucose utilization by the cultures of hepatoma as compared to normal liver would appear to be consistent with the considerably greater production of lactic acid in the former, the differences in lactic acid production pointing to basic metabolic differences between the two with regard to the rate of glycolysis. That the difference is not

due to the uneven oxidation of lactic acid in the two tissues is suggested by the observation that lactate oxidation proceeds as well in hepatoma slices as in liver slices (89,149).

Thus the results point to qualitative differences between hepatoma and normal liver with regard to glucose utilization when cultivated in synthetic medium and the similarity of the former to the liver of newborn rat, from which, however, it differs quantitatively.