Chapter III

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Studies described in the previous chapter pointed to qualitative differences in glucose utilization between the cultures of hepatoma and normal liver, and quantitative differences between the former and newborn liver. The hypothesis that glucose utilization and release may depend entirely on the initial glycogen level was not supported by the observation that fasting liver releases glucose into the culture medium despite its low glycogen content. On the other hand, the suggestion that the qualitative similarity between hepatoma and newborn liver may be due to the fact that both are fast-growing tissues is not supported by the observation that regenerating liver which is also a fast-growing tissue shows a markedly different behaviour. These observations raise the question as to whether there are underlying differences in the activities of the different enzymes concerned with the production and utilization of glucose. Subsequent studies were therefore directed towards a comparison of the activities of some of

these enzymes in the different tissues studied, viz, phosphorylase, phosphoglucomutase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase, and fructose-1,6-diphosphatase.

Experimental

Chemicals

The following chemicals were used in addition to those detailed in Chapter II :-

Glucose-1-phosphate, glycogen, fructose-6-phosphate, and fructose-1,6-diphosphate, from British Drug Houses, Ltd., England; glucose-6-phosphate from Sigma Chemical Co., U.S.A.

Preparation of tissue homogenate

The animals were killed by decapitation and bled. The livers were quickly removed, blotted on filter paper, and chilled in a beaker placed in cracked ice for 5 minutes. The tumor tissues were similarly excised and dissected free of necrotic, hemorrhagic and non-tumorous material to the maximum extent possible. The chilled tissues were minced and homogenized with isotonic KCl or 0.05M sodium bicarbonate in MSE homogenizer for 2 minutes in cold room. The homogenates were centrifuged in Servall refrigerated centrifuge at 500 x g and 0° for 30 minutes and the

supernatants obtained used for the experiments. Unless otherwise specified, 10% homogenates were prepared, dilutions being made where necessary.

The protein content of the enzyme solution was determined after suitable dilution using the method of Warburg and Christian (173).

Enzyme assay

Phosphorylase: Phosphorylase was determined by the method described by Hadjiolov and Dancheva (86).

The substrate mixture contained 0.05M each of glucose-1-phosphate and sodium fluoride and 5.7 mg./ml. of glycogen. The reaction mixture was composed of 0.25 ml. of the substrate; enzyme extract; and water to a volume of 3 ml. The mixture was incubated at 30° for 15 minutes, the reaction stopped by the addition of 2.0 ml. of 10% TCA (Trichloroacetic acid) and the precipitated protein removed by centrifugation. The inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow (174). The blank was prepared by the same procedure except that TCA was added before the addition of enzyme.

A unit of enzyme activity is defined as the amount of enzyme which, under the conditions of the assay, liberates one microgram of phosphorus in 15 minutes, and specific activity, as units per mg. protein.

Phosphoglucomutase: Phosphoglucomutase activity was determined by the method described by Najjar (175).

The reaction mixture consisted of : glucose-1-phosphate, 2.0 micromoles; magnesium sulphate, 0.6 micromoles; cysteine hydrochloride (neutralized), 10 micromoles; enzyme extract; and water to a total volume of 1.0 ml. After incubation for 15 minutes at 30°, the reaction was stopped by the addition of 2.0 ml. of 5N H_2SO_4 and the mixture diluted to 5.0 ml. with water. This was kept in a boiling water bath for 3 minutes and centrifuged. The inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow (174). The blank was prepared by the same procedure except that H_2SO_4 was added before incubation.

A unit of enzyme activity is defined as the amount of enzyme which catalyses the disappearance of one microgram of acid labile phosphorus in 5 minutes under the conditions specified, and specific activity, as units per milligram protein.

Glucose-6-phosphatase : Glucose-6-phosphatase was assayed by the method described by Swanson (176).

The reaction mixture consisted of : maleic acid buffer pH 6.8, 20 micromoles; glucose-6-phosphate, 5.0 micromoles; enzyme preparation; and water to a total volume of 1.0 ml. After incubation for 10 minutes at 37°, the reaction was stopped by the addition of 2.0 ml. of 10% TCA and the tubes chilled and centrifuged. The inorganic phosphate in the supernatant was determined by the method of Fiske and SubbaRow (174). For the blank TCA was added before the addition of enzyme.

A unit of enzyme activity is defined as the amount of enzyme which liberates one microgram of inorganic phosphate in 10 minutes under the conditions specified, and specific activity, as units per milligram protein.

Phosphoglucose isomerase : The method of estimating phosphoglucose isomerase activity was the same as that described by Slein (177) except that the reaction was carried out at pH 7.0.

The reaction mixture consisted of : phosphate buffer, pH 7.0, 25 micromoles; glucose-6-phosphate, 2.0 micromoles;

enzyme extract; and water to a final volume of 2.0 ml. After incubation for 5 minutes at 30°, the reaction was stopped by the addition of HCl (5:1), and the amount of fructose-6-phosphate formed determined by the method described by Roe et al. (178). For the blank, the enzyme was added after addition of HCl.

A unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 0.1 micromole of fructose-6-phosphate in 5 minutes under the experimental conditions, and specific activity, as units per milligram protein.

Glucose-6-phosphate dehydrogenase : The enzyme activity was determined by the method of Glock and Mclean (179).

The reaction mixture consisted of ; glycylglycine buffer, pH 7.6, 50 micromoles; glucose-6-phosphate, 5.0 micromoles; magnesium chloride, 50 micromoles; triphosphopyridine nucleotide, 0.25 micromoles; enzyme extract; and water to a total volume of 3.0 ml. The blank contained all the components except triphosphopyridine nucleotide. The reaction was started by the addition of enzyme and the increase in optical density at 340 mμ read off at one minute intervals against the blank, using

Beckman quartz spectrophotometer.

A unit of enzyme activity is defined as the amount of enzyme which, under the conditions of the assay, causes an increase in optical density of 0.001 per minute, and specific activity, as units per milligram protein.

Fructose-1,6-diphosphatase : The enzyme activity was determined by the method described by Weber and Cantero (180).

The reaction mixture consisted of : glycylglycine buffer, pH 7.0, 100 micromoles, sodium borate, 20 micromoles; fructose-1,6-diphosphate, 5.0 micromoles; magnesium sulphate, 50.0 micromoles; enzyme extract; and water to a total volume of 1.5 ml. The reaction mixture was kept at 37° and one ml. of 5% TCA added to stop the reaction at 0,10 and 20 minutes. The liberated inorganic phosphate was measured by the method of Fiske and SubbaRow (174).

A unit of enzyme activity is defined as the amount of enzyme which liberates one microgram of inorganic phosphate in 10 minutes under the conditions specified, and specific activity, as units per milligram protein.

Results and discussion

Comparative data on the activities of these enzymes in normal, neoplastic, newborn, and regenerating liver are presented in Table 3 on a wet weight basis and in Table 4, in terms of specific activity. Fig. 2 presents the data of Table 4 as percentage of activity in normal liver.

It can be seen from the tables that hepatoma tissue differs significantly from the other tissues studied in respect of the activities of several enzymes, there being a considerable decrease in enzyme level with regard to phosphoglucomutase, glucose-6-phosphatase and fructose-1,6-diphosphatase in terms of both total and specific activities. To this list must perhaps be added phosphoglucose isomerase and also phosphorylase, the former in terms of total activity, and the latter as compared to normal and regenerating but not newborn liver tissue. Glucose-6-phosphate dehydrogenase on the other hand shows an increase in terms of specific activity.

A significant observation that emerges from these results is that the qualitative similarity in glucose utilization between cultures of newborn and neoplastic liver is not reflected in the respective enzyme patterns

Table 3

Comparative data on the activities* of certain enzymes of carbohydrate metabolism in normal, neoplastic, newborn and regenerating liver

Enzyme	Type of liver tissue			
	Normal	Neoplastic	Newborn	Regenerating
Phosphorylase	5400	1605	1200	3598
Phosphoglucomutase	7400	1400	6300	7100
Glucose-6-phosphatase	2000	200	2220	1900
Phosphoglucose isomerase	6600	3600	7600	6000
Fructose-1,6-diphosphatase	2370	650	1510	2425
Glucose-6-phosphate dehydrogenase	8400	8800	7600	10000

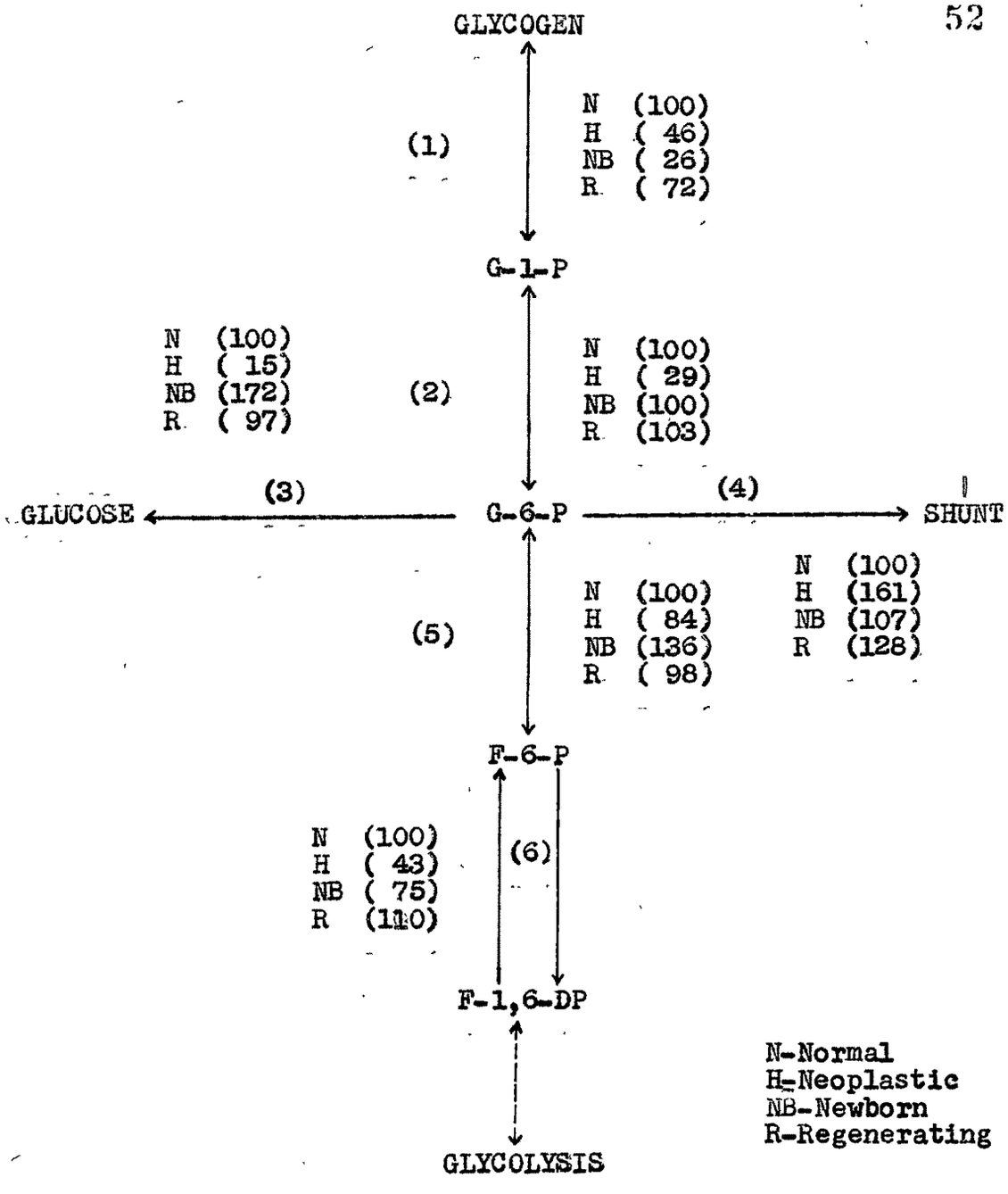
* Expressed as units per gram of wet tissue.

Table 4

Comparative data on the activities* of certain enzymes of carbohydrate metabolism in normal, neoplastic, newborn and regenerating liver

Enzyme	Type of liver tissue			
	Normal	Neoplastic	Newborn	Regenerating
Phosphorylase	25.1	11.6	6.5	17.9
Phosphoglucomutase	34.4	10.1	34.6	35.5
Glucose-6-phosphatase	9.3	1.4	16.0	9.0
Phosphoglucose isomerase	30.7	26.0	41.7	30.0
Fructose-1,6-diphosphatase	11.0	4.8	8.3	12.1
Glucose-6-phosphate dehydrogenase	39.0	63.0	41.7	50.0

* Expressed as units per milligram of protein.



(1)-Phosphorylase

(2)-Phosphoglucomutase

(3)-Glucose-6-phosphatase

(4)-Glucose-6-phosphate dehydrogenase

(5)-Phosphoglucose isomerase

(6)-Fructose-1,6-diphosphatase

Fig. 2. Comparative values for the specific activities of certain enzymes of carbohydrate metabolism in normal, neoplastic, newborn, and regenerating liver tissues.

except with regard to phosphorylase whose relatively low levels in both these tissues would appear to account for their low glycogen content. On the other hand the results are suggestive of qualitative differences between the two with regard to glucose-6-phosphatase.

In view of the demonstrated synthesis of glycogen via uridine diphosphate glucose (181-183), the question may be raised as to how the low levels of phosphorylase can completely determine the extent of glycogen synthesis. However, the normal operation of this pathway must be ruled out in hepatoma because of the very low levels of not only of glycogen synthetase (184) but also of phosphoglucomutase. A similar decrease in phosphorylase and glycogen synthesis has been reported with regard to other varieties of tumor tissue (86,185).

While the considerably high rate of glucose utilization in both newborn and neoplastic cultures is consistent with the high values reported for hexokinase and phosphohexokinase activities in these tissues (89,186), the decrease in phosphatase activity appears to be specific to the tumor tissue. Such a decrease can be considered to favour increased utilization of glucose which together with the increase in specific activity of glucose-6-phosphate dehydrogenase is suggestive of increased operation of the

hexose monophosphate shunt in hepatoma.

The lowered activity of fructose-1,6-diphosphatase together with the reported increased activity of phosphohexokinase (89) would account for the increased formation of lactic acid in tumor tissue. Similar considerations may perhaps be applicable to newborn liver as well.

Thus it would appear from a comparison of the enzyme patterns in normal and neoplastic liver that the decreased activities of phosphorylase and phosphoglucomutase would account for the low glycogen content of the latter whereas the low activities of fructose-1,6-diphosphatase and glucose-6-phosphatase would account for the absence of glucose release and perhaps also for glucose utilization and increase in lactic acid production.

These studies further underline the biochemical uniqueness of tumor tissue from amongst the tissues studied despite the appearance of a qualitative similarity between this and newborn liver with regard to certain nutritional characteristics. Incidentally, these studies have also pointed to the distinguishing characteristics of newborn liver and regenerating liver from normal liver and from each other.

When a comparison is made of enzymatic lesions observed in primary hepatoma with those in other liver tumors, a significant point that emerges is that most of the lesions which are found in the present case have also been reported for Novikoff and Morris hepatomas, the same being found in a more extensive form in the former (94,95).

These studies thus show that those enzymes which channel glucose-6-phosphate into storage or release are greatly diminished in primary hepatoma while those enzymes which channel it into glycolysis are maintained or increased. It would also appear from these studies that the utilization or release of glucose depends on a number of factors such as glycogen content of the tissue, levels of glucose-6-phosphatase and fructose-1,6-diphosphatase, and growth potential of the tissue in vitro.