49. THE METABOLISM OF RAT LIVER DURING CARCINOGENESIS BY BUTTER YELLOW

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THE production of liver tumours in rats by the inclusion of azo-dyes in their diet was first demonstrated by Yoshida [1932; Sasaki & Yoshida, 1935]. In this work the dye used was *o*-aminoazotoluene (2:1:1:4:3-tolueneazoaminotoluene). Later butter yellow (dimethyl yellow; *p*-dimethylaminoazobenzene) was found to produce the same result more quickly and with a higher yield of tumours [Kinosita, 1937]. The whole subject has been thoroughly reviewed by Kinosita [1937].

These tumours were shown [Nakatani *et al.* 1938] to possess the type of metabolism which is, according to the majority of observers, characteristic of tumours in general, viz. a high rate of anaerobic glycolysis and a considerable rate of aerobic glycolysis. Normal rat liver, on the other hand, according to Warburg *et al.* [1924] shows a low anaerobic glycolysis ($Q^{N_2}_{CO_2} \sim 4$) and negligible aerobic glycolysis ($Q^{O_2}_{CO_2} \sim 1$). The Japanese workers further found that during the period preceding the appearance of a tumour the liver showed a progressively increasing anaerobic glycolysis, from the normal value of $Q^{N_2}_{CO_2} \sim 4$ up to $Q^{N_2}_{CO} \sim 12$. The aerobic glycolysis meanwhile remained at its normal low level. When tumours appeared, they showed the type of metabolism usually associated with tumours, while the remainder of a tumour-bearing liver retained its previous metabolism (high anaerobic, low aerobic glycolysis).

In our first experiments on this subject we were unable to obtain consistently the low values of $Q^{N_2}_{CO_2}$ in normal liver which are quoted by Warburg *et al.* [1924] and Nakatani *et al.* [1938]. Our values ranged indiscriminately between 3 and 15. On consulting the literature, we found that we and the Japanese workers had overlooked the work of Rosenthal [Rosenthal & Lasnitzki, 1928; Rosenthal, 1929], which fundamentally changes the nature of the problem. Rosenthal's results would seem to suggest that (1) liver is not able to glycolyse glucose added to the external medium, but only the glycogen contained in the cells, (2) the rate of this autoglycolysis depends on the state of nutrition of the rat, i.e. presumably on the concentration of glycogen in the liver. In starved animals $Q^{N_2}_{CO_2}$ may be as low as 1, in well-fed ones as high as 16.[1]

[1] Rosenthal found that liver slices placed directly in N_2 showed only a small glycolysis, the high values in well-fed rats being shown by slices removed to N_2 after preliminary incubation in O_2. 30 min. incubation in O_2 at either 20° or 37° sufficed to produce the maximum value of $Q^{N_2}_{CO_2}$. We have failed to observe this phenomenon, and our results under conditions of 'direct' anaerobiosis obviously correspond with his after a preliminary aerobiosis. Perhaps there was a greater delay in our case (up to 20 min. from the killing of the rat to the establishment of anaerobiosis), and our slices had in effect been incubated in air at 20° for a sufficient period to raise $Q^{N_2}_{CO_2}$ to its maximum value.

These considerations make the interpretation of the results of Nakatani *et al.* [1938] very difficult, and we decided to study the following points:

(1) The highest value of $Q^{N_2}_{CO_2}$ recorded by Nakatani *et al.* for liver slices before the appearance of a tumour lies well within our normal range. Is there any evidence of an increase of anaerobic glycolysis of the liver during butter yellow feeding?

(2) Normal liver does not glucolyse. Does butter yellow feeding cause the liver to acquire the power of glucolysis at any stage before the appearance of a tumour?

(3) The glycolytic mechanism of all other tumours so far investigated acts only on monosaccharides. Is this also true of the tumours produced in liver?

(4) There are two main types of carcinomata produced in liver by the action of butter yellow, one derived from the liver parenchyma (hepatomata), and the other from bile-duct epithelium (cholangiomata). In normal liver the liver cells predominate to such an extent that the values for the metabolism of the tissue may be taken to represent the metabolism of the liver cells only. The metabolism of normal bile-duct epithelium is therefore unknown. Do liver cells, when they form tumours, show an abrupt change in metabolism from glycogen glycolysis to glucolysis?

This paper provides an answer to the first three of these questions. On account of the breaking off of our experiments in September 1939, the answer to the fourth remains for the present only tentative.[1]

Experimental

Administration of butter yellow to rats. White stock rats, obtained from a dealer, were used. At the start of the experiment they were almost, but not quite, full-grown. The first group were fed on a mixture of wheat, maize and oats, with supplements of green vegetables and water *ad lib.*; in a later group the cereal food was unpolished Indian rice, as there is evidence [Ando, 1938] that the changes develop more rapidly in rice-fed than in wheat-fed animals. There was no significant difference in the yield or rate of appearance of tumours, or in the findings now reported, as between these two experiments.

The dye used was 'dimethyl yellow (Analar)' and was obtained from British Drug Houses. It was dissolved in warm olive oil in a concentration of 3 %; on cooling, a very small amount crystallized out, and the saturated solution was added to, and intimately mixed with, the cereal part of the animals' food in the proportion of 20 or 30 ml./kg.

It is important to point out that the changes in the livers of our animals progressed at a much slower rate than has been described by the Japanese workers. The detailed histology has been fully described elsewhere [Orr, 1940]. Briefly, the earliest changes in the liver parenchyma are of a degenerative type. After a month, microscopical examination shows proliferation of connective tissue in the neighbourhood of the portal tracts. Later there is added to this regenerative proliferation of both liver tissue and bile ducts, in varying proportions. After about 3 months, as a result of all these processes, the liver has a

[1] An attempt since then to amplify our observations has been unsuccessful as a result of the failure of the rats to develop tumours after up to 15 months of butter yellow feeding. The principal difference has been that these rats have been fed on ordinary white bread with greenstuff supplements. In the original experiments the main diet was unpolished rice, or a mixture of wheat, maize and oats. We have not yet found it possible, under present circumstances, to investigate this problem.

granular appearance similar to that of ordinary cirrhosis, and histologically shows marked replacement of the normal lobular architecture by regeneration nodules of parenchyma and trabeculae of granulation tissue; this stage may conveniently be termed 'nodular hyperplasia'. Carcinomata were first found in the 5th month of treatment, the earliest ones being derived from bile duct epithelium (the so-called cholangiomata); tumours of liver cells proper (hepatomata) appeared rather later, but frequently both types of carcinoma were found in the same liver. It was therefore important that the actual tumour whose metabolism was measured should also be subjected to histological examination; this was done in all instances.

Measurement of metabolism. The manometric methods employed were those described by Berenblum *et al.* [1936], but tissue slices were always used. It must be noted that this method of measuring the aerobic glycolysis, unlike that of Warburg [1924], gives only the glucolysis; both anaerobic methods of course measure the whole lactic acid production from whatever carbohydrate substrates may be present. For the determination of the rate of aerobic glycolysis in normal liver, therefore, the chemical method of lactic acid estimation has been used [Friedemann *et al.* 1927]. Manometric and chemical determinations in the same (anaerobic) experiment showed satisfactory agreement, the manometric figures being usually some 10 % higher than the chemical (cf. Table 7).

Estimation of glycogen concentration in liver. The usual method was used, the details being according to Hynd & Rotter [1930].

I. $Q_{CO_2}^{N_2}$ *of normal liver tissue*

As a result of our earliest experiments and consideration of Rosenthal's work, we decided that the estimation of liver glycolysis, without simultaneous

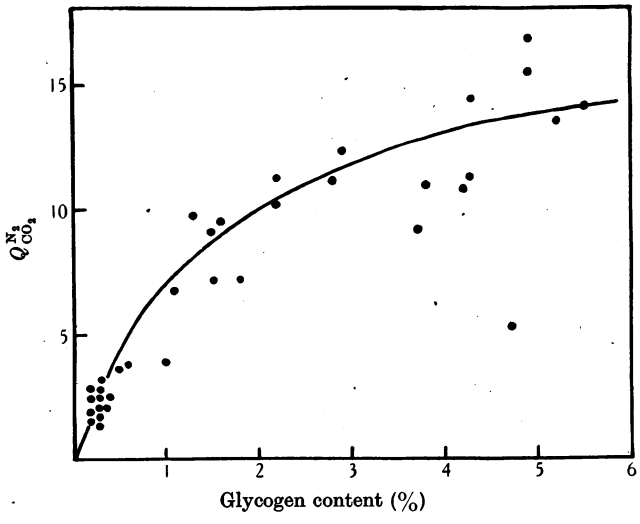

Fig. 1. Rate of glycolysis and glycogen content of normal livers.

determination of the glycogen content of the tissue, yielded results the interpretation of which was necessarily obscure. A number of rats with different liver glycogen concentrations was therefore used to establish the relationship between rate of glycolysis and glycogen concentration. Attempts were made to

produce different glycogen contents by injection of varying amounts of glucose solution into starved rats at appropriate times before killing the animals, but such methods were found to be unreliable. Eventually sufficient liver glycogen values in the range from 1 to 5 % were obtained by random sampling from fed rats, and values from 0·1 to 0·5 % from rats starved for periods up to 48 hr.

The results are shown in Fig. 1. The points are rather scattered, but there is obviously an association between rate of glycolysis and glycogen concentration. It is seen that normal rat liver frequently shows a $Q_{CO_2}^{N_2}$ value of 15 or more, while the highest figure recorded by Nakatani *et al.* [1938] for the liver of rats fed on butter yellow was 11.

It is evident that a comparison of the average values of $Q_{CO_2}^{N_2}$ for a large number of rats of different liver glycogen levels is not a good criterion for determining whether butter yellow feeding induces any change in the rate of glycolysis. In practice, a fairly satisfactory comparison can be made by considering only those cases in which the liver glycogen was 1·5 % or greater. It can be seen from Fig. 1 that from 1·5 to 5 % glycogen the rate of glycolysis increases relatively slowly. Such data for normal livers are shown in Table 1.

Table 1. *Normal liver slices.* $Q_{CO_2}^{N_2}$ *and glycogen concentration*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
16·8	4·9	11·3	2·2	9·4	1·6
15·4	4·9	11·3	4·3	9·1	3·7
14·4	4·3	11·0	3·8	9·1	1·5
14·1	5·5	11·1	2·8	7·3	1·8
13·5	5·2	10·8	4·2	7·1	1·5
12·3	2·9	10·2	2·2	5·2	4·7
			Average	11·1	3·4

II. $Q_{CO_2}^{N_2}$ *of liver tissue from rats receiving butter yellow*

Nakatani *et al.* [1938] related the increase in rate of glycolysis of liver slices solely with the duration of butter yellow feeding. We find that the response to the butter yellow is very irregular; for instance, after some 3 months of feeding, some livers are macroscopically almost normal while others show advanced cirrhosis. In Table 2 we have therefore classified the livers roughly on the basis of the histological results (*v. supra*), under the following headings:

(*a*) Normal.

(*b*) Degenerative changes only.

(*c*) Periportal connective tissue proliferation without macroscopical granularity.

(*d*) Nodular hyperplasia with loss of architecture.

There is clearly no evidence here of a progressive increase in the rate of glycolysis, parallel with the changes produced by the feeding of butter yellow.

In Table 3 we have analysed the data for the 20 livers of Table 2 (*c* and *d*), grouping them according to their glycogen contents, and comparing their glycolytic rates with those of a similar series of 18 livers from normal rats. It will be seen that while in both series there is the expected rise in glycolysis as glycogen content increases, in no case is the glycolysis of the butter yellow livers significantly higher than that of the corresponding normal groups. All these differences fall far below the conventional level of significance (D/S.E. $=2$).

Table 2. $Q_{CO_2}^{N_2}$ *of livers from rats fed with butter yellow*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
(*a*) Livers which after various periods of butter-yellow feeding were histologically normal:					
14·1	5·5	11·1	2·8	9·1	1·5
13·5	5·2	9·8	3·8		
			Average	11·5	3·7
(*b*) Livers showing degenerative changes:					
11·6	3·0	11·0	3·6	6·4	5·0
			Average	9·7	3·9
(*c*) Livers showing periportal changes:					
14·4	4·0	8·7	2·6	6·7	1·9
14·3	4·1	7·9	3·7	6·4	3·7
13·2	5·5	7·7	3·2	4·5	2·8
12·9	3·0	7·5	2·0	3·7	2·1
11·8	3·1				
			Average	9·2	3·3
(*d*) Livers showing nodular hyperplasia:					
16·6	2·6	13·2	5·1	12·8	4·4
14·2	5·3	13·0	4·2	11·0	4·7
13·5	1·7				
			Average	13·5	4·0

Table 3. *Comparison of precancerous livers of butter yellow-fed rats with normal livers of similar glycogen content*

Range of glycogen %	Average $Q_{CO_2}^{N_2}$ ±standard error		
	Normal	Butter yellow	*D*/S.E.
1·5–2·5	9·1±0·5	7·8±2·1	−0·6
2·5–3·5	11·7±0·6	10·4±1·7	−0·7
3·5–4·5	11·3±0·9	11·5±1·4	+0·1
4·5–5·5	13·4±2·0	12·9±0·7	−0·2

III. *Glycolysis in regenerating liver*

Regeneration of surviving parenchyma plays an important part in the alteration of liver structure during cirrhosis, so for comparison we have made some measurements on regenerating livers. After removal of about one-half of the organ, a rat's liver regains its normal weight in about 8 days, and the maximal rate of growth, as estimated by the number of cells in mitotic division, occurs at about the 4th day. A number of livers were therefore studied on the 3rd, 4th or 5th day after the removal of one-half of the liver. The results are given in Table 4, and it is plain the $Q_{CO_2}^{N_2}$ of regenerating liver does not appreciably differ from that of normal liver.

Table 4. *Glycolysis of regenerating livers*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
16·7	4·96	14·9	6·20	13·4	3·32
6·7	2·40	15·1	3·18	14·4	3·95
14·7	4·13	12·3	4·30		
			Average	13·5	3·55

IV. *Absence of glucolysis in liver tissue*

In our series of measurements on normal liver there was apparent in general a slightly greater glycolytic rate in the presence of glucose than in its absence, and this difference was not appreciably altered by butter yellow feeding (Table 5). At the same time, we do not believe that this finding is in disagreement with the observations of Rosenthal & Lasnitzki [1928] and Dickens & Greville [1932] that normal liver does not glucolyse, because the differences are small, not invariable, and can be interpreted in more than one way (*v. infra*). The results of Rosenthal [1929] are in agreement with ours in showing a slight increase of $Q^{N_2}_{CO_2}$ in presence of glucose, though he makes no comment on this fact. A curious point is that rat liver readily attacks fructose, and that fructolysis is slight in starved animals and considerable in fed ones [Rosenthal, 1930; Dickens & Greville, 1932].

Table 5. *Glucolysis in liver tissue*

$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$	
Glucose absent	Glucose present	Glucose absent	Glucose present	Glucose absent	Glucose present
(*a*) Normal and regenerating:					
2·1	2·8	2·2	2·8	5·8	7·4
0·5	1·3	1·2	1·7	3·7	5·0
9·3	8·0	8·2	7·1	12·3	12·0
4·8	5·5	6·7	7·0	15·1	17·6
16·7	17·6	5·8	7·1	2·7	3·5
3·0	3·7	1·8	2·4	3·1	3·2
2·3	3·1	0·8	2·3	14·1	12·7
13·5	16·2	9·7	8·4	11·1	11·1
9·1	6·9	9·3	8·0	8·2	7·1
			Average	6·8	7·1
(*b*) Degenerative changes:					
11·0	12·7	11·6	11·3	6·4	9·4
			Average	9·7	11·1
(*c*) Periportal changes:					
14·3	14·5	7·7	9·4	7·9	8·7
14·4	14·2	6·7	4·8	8·7	8·5
6·4	5·2	3·7	7·7	13·2	17·0
4·5	5·2	7·5	14·8	12·9	13·1
11·8	14·3	1·8	4·5	3·0	3·4
			Average	8·5	9·7
(*d*) Nodular hyperplasia:					
13·0	16·9	12·8	16·6	13·2	14·2
11·0	13·2	13·5	15·2	1·9	4·6
2·0	4·4	14·2	15·4	3·1	7·3
16·6	12·2	4·5	7·7		
			Average	9·6	11·6

It is clear from these figures that there is no striking appearance of glucolysis during the feeding of butter yellow to rats, indeed it would require a very large number of observations to support a claim that there was any significant amount of glucolysis at all.

The same result is found with regenerating livers (Table 6).

Aerobically also liver slices glycolyse only glycogen. A large number of manometric measurements of aerobic glucolysis gave for $Q^{O_2}_{CO_2}$ an average figure

Table 6. *Glucolysis in regenerating livers*

$Q_{CO_2}^{N_2}$	
Glucose absent	Glucose present
16·7	17·6
5·8	7·1
15·1	17·6
4·8	5·5
6·7	7·0
Average 9·8	11·0

of $1·1 \pm 1·1$, which is not significantly different from zero, while chemical estimation of the total aerobic glycolysis (lactic acid formation) gave the results in Table 7 (the anaerobic results for the same livers are given for comparison, simultaneous chemical and manometric estimations being given side by side to show the degree of agreement).

Table 7. *Glycolysis of normal liver slices*

The paired values in each column are derived from duplicate experiments with material from the same source.

$Q_{CO_2}^{N_2}$ (manometric)		$Q_{CO_2}^{N_2}$ (chemical)		$Q_{CO_2}^{O_2}$ (chemical)	
8·5	9·3	8·7	10·0	1·9	1·8
4·7	4·6	5·6	5·2	1·9	1·7
10·2	10·7	9·4	10·0	2·2	2·4
4·3	4·6	4·3	4·5	1·6	1·5
4·5	4·5	5·3	4·4	1·8	1·9
2·9	3·4	2·4	2·8	1·5	1·3
10·5	10·8	10·4	10·7	1·9	—
8·3	9·5	8·1	9·7	1·9	1·9

V. *The metabolism of the tumours induced by butter yellow*

Owing to the premature end of our experiments, our data on the metabolism of butter yellow tumours are scanty. They are collected in Table 8.

Table 8. *Metabolism of liver tumours*

	$Q_{CO_2}^{N_2}$		$Q_{CO_2}^{O_2}$
Type of tumour	Glucose absent	Glucose present	(glucolysis only)
Cholangioma	—	5·5	2·5
,,	—	13·2	5·2
,,	—	13·0	5·4
Hepatoma	—	20·8	8·6
,,	0·0	17·0	6·0
,,	1·9*	8·0*	—
,,	—	11·4	3·2

* This was not a single discrete tumour, but a number of separate nodules. The slices were cut so as to include as much tumour tissue as possible, but they were contaminated with some liver tissue.

Only two results are available which show directly the presence of anaerobic glucolysis in hepatomata. However, in the cases of the three cholangiomata and of the other two hepatomata there is a considerable aerobic glycolysis. As was stated before, our method of measuring aerobic glycolysis manometrically

excludes the lactic acid production from any substrate except the added glucose, so these results all support the view that glucolysis does occur, both in cholangiomata and in hepatomata.

Discussion

From our results we may attempt to answer the four questions mentioned in the introduction (p. 480):

(1) There is no evidence of an increase in the rate of anaerobic glycolysis of liver tissue of rats at any stage of feeding of butter yellow. The increase found by Nakatani *et al.* [1938], unless there is a difference in behaviour between the strains of rats used by them and by us, must have been due to chance. They found an increase of $Q_{CO_2}^{N_2}$ from 4 in normal livers to 12 in cirrhotic livers, while we find that $Q_{CO_2}^{N_2}$ may lie anywhere between 2 and 16 in any rat, according to the glycogen concentration in the liver.

(2) Liver tissue does not acquire an increased power of glucolysis at any stage, prior to actual neoplasm, of butter yellow feeding. The presence of glucose causes an average increase of about 1 in the value of $Q_{CO_2}^{N_2}$, which increase is independent of the absolute value of $Q_{CO_2}^{N_2}$, i.e. is independent of the glycogen content. Among possible explanations of this are (*a*) that liver cells have a slight power of glucolysis, (*b*) that a small proportion of the cells of the liver (e.g. possibly the bile-duct epithelium as distinct from the liver cells) are glucolytic, or (*c*) that the presence of glucose increases the rate of glycogen breakdown.

(3) All the liver tumours we have studied possessed the power of glucolysis. In the two cases in which the point was specially studied, it was found that there was no autoglycolysis, i.e. no glycolytic breakdown of glycogen, and only glucose was attacked. However, histologically the tumour tissue fixed in alcohol was free from glycogen, so there is no possibility of testing whether the tumours would attack glycogen if that substrate were present.

(4) The answer to this important question depends on the results of only four experiments, so we give it only tentatively, and hope to obtain further data in confirmation later. It appears probable, however, that in the transition to malignancy there occurs abruptly a complete change in the type of metabolism of the liver cells, viz. the sole substrate used for glycolysis changes from glycogen to glucose.

Warburg was the first to show a qualitative difference in metabolism between normal and tumour tissues, his results showing that tumour metabolism was characterized by a high rate of glycolysis which was only partially suppressed by oxygen. Since then various normal tissues have been shown to have a similar type of metabolism, e.g. testis (rat), retina (rat) and hyperplastic tonsil (man) [Warburg *et al.* 1924], embryonic lens of rat [Fujita, 1928], kidney medulla in several species [György *et al.* 1928; Dickens & Weil-Malherbe, 1936] and bone marrow of rabbit [Orr & Stickland, 1938], so its specificity to tumours is by no means complete. Recently the criticism has been made [Berenblum *et al.* 1940] that the metabolism of a tumour has in no case been compared with that of the normal tissue from which it is derived, because the normal tissues in question cannot be obtained in sufficient quantity for use with the apparatus so far available. With their new micro-apparatus they have shown that skin epithelium has a metabolism qualitatively the same as that of tumours derived from it, and suggest that the same may be true of all tumours.

Our present results, if confirmed, prove on the contrary that liver cells at any rate do show a sudden change in the type of their metabolism when they form tumours.

SUMMARY

Contrary to the findings of Nakatani *et al.* [1938] we have not observed any change in the glycolytic metabolism of liver tissue in the precancerous phase of butter yellow treatment.

On the other hand, the tumours produced by this agent show a qualitative difference in metabolism from that of normal and cirrhotic liver, the substrate for glycolysis being glycogen in liver tissue and glucose in the tumours derived from it.

The tumours show an aerobic glucolysis equivalent, on the average, to rather more than one-third of the anaerobic glycolysis.

REFERENCES

Ando (1938). *Gann*, **32**, 252.
Berenblum, Chain & Heatley (1940). *Amer. J. Cancer*, **37**, 367.
—— Kendal & Orr (1936). *Biochem. J.* **30**, 709.
Dickens & Greville (1932). *Biochem. J.* **26**, 1546.
—— & Weil-Malherbe (1936). *Biochem. J.* **30**, 659.
Friedemann, Cotonio & Schaffer (1927). *J. biol. Chem.* **73**, 335.
Fujita (1928). *Biochem. Z.* **197**, 175.
György, Keller & Brehme (1928). *Biochem. Z.* **200**, 356.
Hynd & Rotter (1930). *Biochem. J.* **24**, 1390.
Kinosita (1937). *Trans. Soc. Path. jap.* **27**, 665.
Nakatani, Nakano & Ohara (1938). *Gann*, **32**, 240.
Orr (1940). *J. Path. Bact.* **50**, 393.
—— & Stickland (1938). *Biochem. J.* **32**, 567.
Rosenthal (1929). *Biochem. Z.* **207**, 263.
—— (1930). *Biochem. Z.* **227**, 354.
—— & Lasnitzki (1928). *Biochem. Z.* **196**, 340.
Sasaki & Yoshida (1935). *Arch. path. Anat.* **295**, 175.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— Posener & Negelein (1924). *Biochem. Z.* **152**, 309.
Yoshida (1932). *Proc. imp. Acad. Tokyo*, **8**, 464.