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Permeability of the Liver Cell Membrane to Quinolinate

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Quinolinate was taken up by both rat and guinea-pig liver cells. Equilibrium was reached after approx. 20min with rat cells, but guinea-pig cells had not achieved a steady state after 60min. There was no evidence to suggest that quinolinate is rapidly metabolized by either species. The concentrations of quinolinate attained in rat and guinea-pig cells after short periods of incubation with 0.5mM-quinolinate did not inhibit gluconeogenesis. These results raise further doubts as to the mechanism of quinolinate action in liver.

Lardy and co-workers have shown that gluconeogenesis in the isolated perfused rat liver is inhibited by tryptophan (Veneziale *et al.*, 1967); synthesis from alanine is almost totally abolished in the presence of 2.4mM-L-tryptophan. The kinetics of inhibition suggest that a metabolite, rather than the amino acid itself, is responsible for the effect. Of the range of metabolites tested as potential inhibitors, quinolinate was found to be the most effective. The locus of action of both tryptophan and quinolinate has been identified as phosphoenolpyruvate carboxykinase [GTP–oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32]. Quinolinate inhibits phosphoenolpyruvate carboxykinase *in vitro*, in a manner non-competitive with oxaloacetate and with a K_i of 5×10^{-4}M (McDaniel *et al.*, 1972). Lardy's group have suggested that Fe^{2+} ions may be important for enzyme activity (Snoke *et al.*, 1971; Bentle & Lardy, 1976) and that the effectiveness of quinolinate may be due to its affinity of this ion.

The discrepancy revealed by the relative effectiveness of quinolinate as an inhibitor in cell-free systems and the higher concentrations necessary in perfusion experiments (>2mM) has been rationalized in terms of poor penetration of this compound across the hepatic plasma membrane. Thus McDaniel *et al.* (1972) have claimed that a cell:medium concentration ratio of 1:10 is present, on the basis of experiments reported by Hagino *et al.* (1968). An earlier report (Ijichi *et al.*, 1966) also claims limited uptake of quinolinate by the liver. Re-examination of the data of Hagino *et al.* (1968), however, suggests that quinolinate may actually penetrate the liver cell quite effectively. Later studies have, in addition, indicated that quinolinate may not in fact act in the manner proposed by Lardy and co-workers (Spydervold *et al.*, 1974).

Since the interpretation of the effects, or absence of effects, of quinolinate depend upon accurate knowledge of the permeability of the liver-cell membrane to this metabolite, we have investigated the phenomenon in hepatocytes derived from both rats and guinea pigs. The results indicate that quinolinate indeed penetrates more readily than hitherto envisaged.

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Materials and Methods

Animals

Male Sprague–Dawley rats (200–250g) and Dunkin–Hartley guinea pigs (300–400g) were used throughout. Unless otherwise specified, all animals were deprived of food for 48h before cell preparation. Diabetes was induced by intravenous injection of 60mg of alloxan/kg body wt.; animals were used 48h later.

Chemicals

[6-^{14}C]Quinolinic acid and [^{3}H]inulin were from The Radiochemical Centre, Amersham, Bucks., U.K. Glucose oxidase and glucose peroxidase were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Other enzymes were from the Boehringer Corp. (London), Lewes, Sussex, U.K. Bovine serum albumin was obtained as a 20% solution from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K., and was freed of fatty acids and other materials by the method of Chen (1967). PCS scintillation fluid was from Hopkin and Williams, Chadwell Heath, Essex, U.K. All other chemicals, of the purest grade available, were from standard suppliers.

Procedures

Isolated liver cells were prepared from both rats and guinea pigs by procedures outlined elsewhere (Elliott *et al.*, 1976). In quinolinate-uptake studies 1ml portions of cell suspensions (containing approx. 20mg dry wt. of tissue) were added to 4ml of buffer

(Krebs & Henseleit, 1932) supplemented with 2% (w/v) bovine serum albumin and gassed with O_2/CO_2 (19:1), in 50ml Erlenmeyer flasks stoppered with rubber bungs. Incubations were carried out under O_2/CO_2 (19:1) at 37°C; the flasks were shaken at 100 oscillations/min in Dubnoff-type shaking water baths (Mickle Engineering, Gomshall, Surrey, U.K.). L-Lactate (final concn. 10mM) was added 10min after the start of incubation. A further 20min later 0.25μCi of sodium [6-^{14}C]quinolinate (final concn. 0.5mM) was added. At various times after this addition, 2.5μCi of [^{3}H]inulin (specific radioactivity 0.25μCi/mg) was added to each flask; cells were immediately separated from incubation medium by using the small size of tube described by Hems *et al.* (1975). Portions of both cell and medium fractions were analysed for ^{14}C and ^{3}H content in PCS scintillator 'cocktail' with a Packard model 3375 liquid-scintillation spectrometer. Counts were corrected for quenching by internal standardization. In experiments to investigate the effect of quinolinate on gluconeogenesis, 0.3ml portions of cell suspensions were added to 1.7ml of buffer (as above) in 20ml silicone-treated glass scintillation vials stoppered with Subaseals (Laboratory Apparatus and Glassblowing Co., Dewsbury, Manchester, U.K.). Incubation and gassing conditions were as described above, except that cells were preincubated with various concentrations of quinolinate for 40min before addition of lactate (final concn. 10mM). Rates of gluconeogenesis were measured between 30 and 90min after the addition of substrate, and were linear over this period.

The possibility of the conversion of quinolinate into other metabolites was investigated as follows. Incubations of liver cells with 10mM-lactate and 0.5mM-[6-^{14}C]quinolinate were terminated, at various times up to 30min after addition of quinolinate, with 2% (w/v) $HClO_4$ (final concn). After centrifugation, samples were adjusted to 1mM-HCl and heated at 100°C for 60min. This procedure hydrolyses nicotinamide nucleotides to nicotinic acid. After cooling, the total volume of each was added to a column of acid-washed charcoal (Norit A; 20mg in columns 4mm diam.) and was washed through with 2ml of water. The columns were eluted with 5ml of ethanol/aq. NH_3 (sp.gr. 0.880)/water (9:1:10, by vol.) containing 0.5mg each of quinolinic acid and nicotinic acid. Eluates were dried down at 75°C in a current of air, and solids were redissolved in 50μl of water. Samples, together with quinolinate and nicotinate markers, were spotted on silica thin-layer plates (Eastman Kodak Co., Kirkby, Liverpool, U.K.); the chromatograms were developed in $CHCl_3$/acetic acid/methanol/water (13:4:2:1, by vol.) (Haworth & Walmsley, 1972). Spots were viewed under shortwavelength u.v. light. Each sample chromatogram was divided into small strips between the origin and the solvent front; the silica from each such strip was transferred to a scintillation vial and counted for radioactivity as described above.

The binding of quinolinate (0.5 and 5mM) to 2% bovine serum albumin in incubation medium was measured by equilibrium dialysis (Lipsett *et al.*, 1973). Samples (2ml) of [6-^{14}C]quinolinate in medium containing 2% albumin were dialysed in triplicate with shaking against 1ml of Krebs–Henseleit (1932) bicarbonate buffer at 37°C for 3½h. Samples from both protein and non-protein compartments were counted for radioactivity in PCS scintillator as described above.

Glucose (Bergmeyer *et al.*, 1974*a*), aspartate (Bergmeyer *et al.*, 1974*b*) and malate (Gutman & Wahlefeld, 1974) were measured by standard techniques.

Results and Discussion

Table 1 shows that quinolinate uptake in rat liver cells obeys simple kinetics and reaches a steady-state value after approx. 20min of incubation; this time course is remarkably similar to that found for glucose uptake by isolated cells (Kletzien *et al.*, 1976).

In their perfusion experiments Veneziale *et al.* (1967) did not find any inhibition of gluconeogenesis from lactate with 0.5mM-quinolinate; the results of Söling *et al.* (1970) are also consistent with this view. Table 2 shows that inhibition by quinolinate is observable only at concentrations above 1mM. There was no clear difference between the response to quinolinate of cells from animals deprived of food for 48h and an alloxan-diabetic animal. In one experiment, the concentrations of malate and aspartate were found to be unchanged in the presence of 0.5mM-quinolinate, but increased from 1.4 and 1.2nmol/mg dry wt. to 3.6 and 5.4nmol/mg dry wt. respectively with 5mM-quinolinate. These changes are similar to those noted by Williamson *et al.* (1971) and Spydervold *et al.* (1974). As a comparison, the corresponding values with 0.5mM-tryptophan (a concentration which gives a pronounced inhibition of gluconeogenesis from lactate) were 3.4 and 5.7nmol/mg dry wt. respectively.

The time course of the inhibition of glucose formation from lactate by quinolinate was investigated in an experiment in which quinolinate (5mM) was added to cells synthesizing glucose linearly from 10mM-lactate. Although the concentration of quinolinate was different in the two experimental situations, it was noticeable that the time course of the onset and increase of quinolinate inhibition of gluconeogenesis parallelled that of quinolinate uptake. In our experiments the concentration of quinolinate reached 0.1mM after only 2min. Assuming ferrous quinolinate to be the inhibitory species (McDaniel *et al.*, 1972), and that the prevailing steady-state cytoplasmic

Table 1. *Uptake of quinolinate by rat and guinea-pig liver cells*

Measurements at each time point were performed in duplicate in each experiment. All values are corrected for cross-contamination of cells with medium (from [^{3}H]inulin measurements), and are means±S.E.M. for four separate experiments, except where indicated in parentheses. Concentrations are based on the assumption that 1g of cells contains 700μl of intracellular water. Other conditions were as described in the Materials and Methods section; the initial concentration of quinolinate was 0.5mM.

Time after addition of quinolinate (min)	Rat		Guinea pig	
	Quinolinate in cells (μM)	Concentration ratio (medium:cells)	Quinolinate in cells (μM)	Concentration ratio (medium:cells)
1	44±9	13.0±2.7	6	86 (1)
2	103±24	6.3±2.1	23	31 (2)
5	—	—	43	11.6 (1)
10	337±55	1.6±0.3	67±12	8.8±2.1
20	373±60	1.4±0.3	153±22	3.5±0.6
30	462±94	1.2±0.3	241±33	2.2±0.3
45	—	—	322	1.6 (1)
60	—	—	336	1.5 (1)

Table 2. *Effect of quinolinate on glucose production from* L-*lactate in hepatocytes from rats and guinea pigs*

Cell incubations were as described in the Materials and Methods section. Cells were preincubated for 40min with quinolinate before addition of lactate. Rates were measured from 30 to 90min after addition of 10mM-lactate. Rates are expressed, where appropriate, as means±S.E.M. with the number of observations in parentheses. Rates from experiments with 10mM-lactate were 198±28 (3), 328 and 117±12 (6) nmol of glucose/h per mg dry wt. for cells from rats deprived of food for 48h, the diabetic rat and guinea pigs deprived of food for 48h respectively.

Quinolate concn. (mM)	Glucose production (% of uninhibited rate)		
	Unfed rats	Diabetic rat	Unfed guinea pigs
0	12±3 (3)	7	
0.05		102	
0.1	106	102	104±10 (5)
0.25	108	103	
0.5	99±1 (3)	102	98±14 (4)
1.0	82	91	92±10 (4)
2.0		60	
5.0	19±3 (3)	36	61± 6 (6)
10.0		14	50±10 (4)

oxaloacetate concentration is similar to the K_m for phosphoenolpyruvate carboxykinase (Hanson & Garber, 1972; Pogson & Smith, 1975), 0.1mM-quinolinate should decrease the flux through this enzyme by approx. 33%. The degree of inhibition should rise to more than 70% after a further 20min incubation. Taking the alternative value for the non-competitive inhibition of the enzyme by 'free' quinolinate (0.5mM; McDaniel *et al.*, 1972), the extent of inhibition at 2 and 20min respectively would be expected to be 17 and 50%.

The extent of the inhibition would, of course, be lessened by increases in oxaloacetate concentration. Such increases were found with higher concentrations of quinolinate by Veneziale *et al.* (1967), but there is disagreement as to whether similar changes occur in the livers from rats pretreated *in vivo* with quinolinate (Williamson *et al.*, 1971; Spydervold *et al.*, 1974), although different conditions prevailed in these two studies. Spydervold *et al.* (1974) attribute the effects of quinolinate in their experiments to interaction with anionic transport systems in the mitochondrial membrane rather than with phosphoenolpyruvate carboxykinase. In view of the difficulties arising from the assumptions implicit in calculations of oxaloacetate concentrations and distributions within the cell, the resolution of this point must depend on more direct methods of measurement.

It seems clear, nevertheless, that the above mechanisms for quinolinate inhibition may not apply in any simple sense in whole tissue. The need for higher concentrations of quinolinate for effective inhibition of gluconeogenesis suggests either that tryptophan (Veneziale *et al.*, 1967) must be converted very rapidly into quinolinate or that one or more other metabolites may also be implicated.

Returning to Table 1, it is apparent that cells from guinea-pig liver exhibit a lower capacity for quinolinate uptake. The time for half-maximal uptake is approx. 30min, compared with 10min for rat cells; even after 60min the ratio of medium to intracellular quinolinate concentrations remains well above unity. This may explain, in part at least, the lower sensitivity of glucose synthesis to exogenous quinolinate in the isolated perfused guinea-pig liver (Table 2; Söling *et al.*, 1970).

The observation that the final value of the ratio of medium to cell quinolinate remains above 1.0 is consistent with the previous observation that quino-

linate does not penetrate the inner mitochondrial compartment (Söling *et al.*, 1970). Although quinolinate is known to be converted into nicotinamide mononucleotide in liver, by the enzyme quinolinate phosphoribosyltransferase (EC 2.4.2.-) (Gholson *et al.*, 1964), it is also found to accumulate in the livers of both untreated and tryptophan-loaded rats (McDaniel *et al.*, 1973). Since our results are consistent with the attainment of a steady state and reveal no evidence of an accumulation against the concentration gradient (i.e. medium [quinolinate]:cell [quinolinate] <1), it would seem that further metabolism of quinolinate is at most very restricted over the time course studied.

This contention is supported directly by the demonstration that the total ^{14}C in extracts from cells incubated for up to 30min with labelled quinolinate plus lactate is absorbed by charcoal. This indicates that no low-molecular-weight non-aromatic products are formed during the 30min period. Further, t.l.c. reveals that more than 97% of the total counts remain associated with quinolinate. This low rate of quinolinate metabolism has also been noted by Hagino *et al.* (1968). It may be explained by the low K_I for NAD^+ ($<10^{-4}$M; Gholson *et al.*, 1964) of quinolinate phosphoribosyltransferase in cells where the prevailing NAD^+ concentration is above 1mM.

The possibility that experimental design and interpretation might be complicated by binding of quinolinate to fatty acid-free albumin was investigated by equilibrium dialysis. The extent of such binding to albumin in incubation media was found, however, to be immeasurably small at both 0.5 and 5mM-quinolinate.

Söling & Kleineke (1976) have studied quinolinate uptake by perfused livers from rats and rabbits, also using [^{3}H]inulin as an extracellular space marker. We have found, however, that, when [^{3}H]-inulin is present during the incubation period, a significant proportion becomes associated with the cell fraction. Thus the ratio of [^{3}H]inulin in cells pre-treated with labelled inulin for 15min to that in cells treated as described in the Methods and Materials section is 1.4 ± 0.15 (mean$\pm$S.E.M. of four determinations). This may be a consequence of non-specific binding of inulin to the plasma membrane or uptake into the liver cell. Incorporation of inulin into lysosomes of isolated cells has been found (C. H. Wynn, personal communication).

The finding by Söling & Kleineke (1976) that rabbit liver is considerably less permeable to quinolinate than is rat liver, taken with the results of the present work, suggests that quinolinate should be used with caution as a metabolic inhibitor in species other than the rat.

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