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BIOCHEMICAL EFFECTS OF BERBERINE

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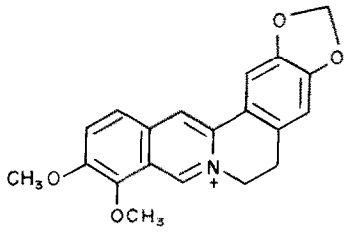
Abstract—The benzodioxoloquinolizine alkaloid berberine inhibited the biosyntheses of DNA, RNA, proteins and lipids, as well as the oxidation of glucose [^{14}C] to $^{14}CO_2$, when incubated with S180 cells *in vitro*. The synthesis of proteins and of RNA was the most sensitive of these parameters to the action of berberine. Only marginal inhibition of protein biosynthesis occurred *in vitro* when both drug and glycine [1-^{14}C] were injected. This difference was reflected in the failure of berberine to inhibit the growth of S180 in mice, despite an inhibitory action in culture. The discrepancy may involve the effects of glucose. When levels of glucose similar to those reported in biological fluids were added to incubation media *in vitro*, the inhibition of protein biosynthesis by berberine was prevented. Glucose and berberine showed mutual antagonism for uptake by S180 cells. Phlorizin, an inhibitor of the active transport of glucose, inhibited the uptake of berberine. Inhibition by berberine of the biosynthesis of macromolecules may reflect such primary actions as inhibition of glucose utilization and interaction with nucleic acids.

Berberine is a benzodioxoloquinolizine alkaloid that is isolated from the Golden seal, *Hydrastis canadensis* L., a member of the family Ranunculaceae; it also occurs in many other plants including the *Berberis* species (Berberidaceae) and *Arcangelisia flava* (Menispermaceae) [1]. In its structure (Fig. 1) and in many of its reported pharmacological actions, berberine shows resemblances to the benzylisoquinoline and aporphine



Berberine

Fig. 1. Structural formula of berberine.

alkaloids thalicarpine, *d*-tetrandrine and papaverine [2]. thus, berberine exhibits a depressive action on excitable tissue, perhaps through inhibition of depolarization and repolarization, and this leads to hypotension; anticholinesterase action has been reported also [3]. Berberine is a more potent inhibitor of the growth of KB cells in culture than is thalicarpine [4]. However, its cytostatic activity against Ehrlich ascites and a lymphomatous ascites tumor is manifested only in culture and not when the tumors are growing in mice [5]. The drug has received some clinical use in the treatment of diarrheas and dermal leishmaniasis, particularly in India [3].

The present report is part of a comparative study of the biochemical interactions of this group of alkaloids. Earlier work was concerned with thalicarpine and *d*-tetrandrine [6, 7].

MATERIALS AND METHODS

Acetate[2-^{14}C], uridine[5, 6-^{3}H], glycine[1-^{14}C], glucose[U-^{14}C] and thymidine[methyl-^{3}H] were purchased from the New England Nuclear Corp. Calf thymus DNA type I, polycytidylic, polythymidylic, polyguanylic, polyadenylic, and polyuridylic acids were purchased from the Sigma Chemical Co., St. Louis, MO. Berberine also was obtained as the hydrochloride from the Sigma Chemical Co.

Sarcoma 180 tumor in the ascites form was maintained in Swiss white mice (CD-1; Charles River Breeding Laboratories, North Wilmington, MA) and harvested 4–6 days after intraperitoneal inoculation (2×10^6 cells). Ascitic fluid was separated by centrifugation and erythrocytes were lysed by suspending the cell pellet in 4 vol. of distilled water. Isotonicity was restored with 4.5% saline, and, after further washing with isotonic saline, the cell pellet was suspended in a Krebs phosphate buffer containing in 125 ml: 0.86 g NaCl; 0.046 g KCl; 0.0073 g $CaCl_2$; 0.0076 g $MgSO_4.7H_2O$, 0.286 g NaH_2PO_4, and 0.32 ml of 1 M HCl, pH 7.4, with 12% dialyzed calf serum [8]. Assays of the inhibition of the growth of S180 ascites in mice were carried out using groups of five animals each. Mice were inoculated intraperitoneally with tumor and drug treatments started 24 hr later and given daily for 5 days. Survival times and weight changes were recorded. Study of the effects of berberine on the growth of S180 cells *in vitro* was carried out in suspension cultures, using Eagle's minimal medium supplemented with glutamine, amino acids, salts, and calf serum. cell numbers were measured with the use of a Coulter counter model A, or by microscopic examination with a hemocytometer.

The synthesis by S180 cells *in vitro* of nucleic acids, residual proteins and lipids was measured by incorporation of thymidine[methyl-^{3}H] (1 μCi; 3 Ci/m-mole), glycine[1-^{14}C] (1 μCi; 44.9 mCi/m-mole) and sodium

acetate-[2-^{14}C] (2.5 μCi; 50 mCi/m-mole), respectively, using procedures that have been described previously [7, 9, 10]. Incubation volumes were 4 ml (with the buffer and serum mentioned above), and about 2×10^7 cells were added per flask. A time course of 30 min was used after an initial preincubation with drug, but no tracer, for 15 min. Oxidation of glucose was measured by incubating cells with glucose[U-^{14}C] (0.05 μCi; 55 mCi/m-mole) in 3 ml of serum-buffer in Warburg flasks. The reaction was stopped by tipping in perchloric acid (0.3 ml; 1 M) from the side arm; $^{14}CO_2$ released during the incubation was collected on filter paper moistened with 0.1 ml of 1 M NaOH in the center well.

For experiments with S180 *in situ* in mice, the animals received i.p. injections of berberine (10 mg/kg) followed 30 min later by glycine[1-^{14}C] (2 μCi; 44.9 mCi/m-mole) or thymidine[methyl-^{3}H] (2 μCi; 6.7 Ci/m-mole). After 30 min for metabolic utilization, the cells were harvested and treated as for the studies done *in vitro*. Data were standardized on the basis of protein, as determined by the biuret reaction, with bovine serum albumin fraction V as a standard [11].

Interaction of berberine with DNA and synthetic polynucleotides was studied by equilibrium dialysis in 0.03 M NaCl solution (pH 6) as described previously [6]. The experiments were carried out at room temperature and in the dark. Absorbance was read at frequent intervals with a Zeiss PMQ II spectrophotometer, using a wave length of 347 nm. Additionally, in the case of DNA, the hypochromic spectral shift at 347 nm was measured as a solution of 2 μg/ml of berberine was allowed to interact with DNA at concentrations from 1 to 150 μg/ml. A Beckman DBG spectrophotometer with a 10 inch recorder was used in these experiments, and the areas of the peaks traced out were measured by cutting them out and weighing them.

For studies of the uptake of berberine and of glucose[^{14}C] by S180 cells *in vitro*, conditions were similar to those described for the incorporation of labeled precursors into macromolecules. At 0, 5, 10 and 20 min, samples were layered over 3.5 ml of 0.25 M sucrose in Shevky–Stafford and McNaught centrifuge tubes and spun at 1600 *g* for 3 min. The supernatant fraction was removed by aspiration and the walls of the tube were dried carefully while the tube was inverted. In the case of the uptake of glucose[^{14}C], the cell pellet was resuspended in 0.2 ml water, transferred to a vial with fluor, and the radioactivity counted in a Packard Tri-Carb liquid scintillation spectrometer. In the case of unlabeled berberine, the cell pellet was resuspended in 0.25 ml water and 5-μl samples were spotted on Whatman No. 3 MM paper. After drying, the paper was stuck to a thin-layer chromatography plate and scanned with a Turner 111 fluorometer equipped with a Camag scanning adaptor. A number 8 filter was used for emission and a 7–60 filter for excitation. Nanogram quantities of berberine could be measured by this method even with a 10 per cent reduction filter to reduce the emission.

RESULTS

Inhibition of tumor growth. Berberine exerted a marked but short-lived inhibitory action on the growth of S180 cells in culture (Fig. 2). However, when given to tumor-bearing mice by daily injections at 2.5, 5, 10, 15 and 20 mg/kg, there was no prolongation of survival. Rather, the life span decreased with increasing doses, and at 15 and 20 mg/kg only three injections were tolerated by the mice.

Inhibition of biochemical activity. In a survey of the effects of two concentrations (4 and 40 μ/ml) of berberine on various biochemical processes, it was evident that the drug interfered with all of them (Table 1). However, the syntheses of protein and RNA were the parameters most sensitive to drug. It is likely that, at the highest drug level (40 μg/ml), inhibition of respiration, shown to result directly from interaction with mitochondria, would cause most biochemical activities to decrease. Measurements of the amounts of total labeled percursor within the cells, an indication of rate of uptake, showed no effect of berberine at levels up to 1 μg/ml and only a maximum of 40 per cent inhibition at 40 μg/ml. Nucleic acid and protein syntheses were examined in greater detail. Dose–response curves of the type shown in Fig. 3 were obtained for the inhibition of thymidine[^{3}H] and glycine[^{14}C] incorporation *in vitro*. *In vivo*, however, treatment of tumorbearing mice with 10 mg/kg of berberine 30 min before injection of radiolabeled thymidine or glycine had only minimal effects on the incorporation of these precursors into

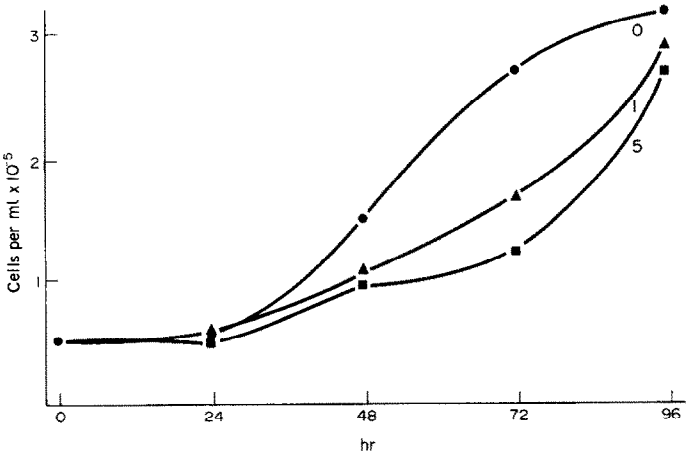


Fig. 2. Growth of S180 cells in culture as a function of berberine concentration. Key: (●——●) control; (▲——▲), berberine, 1 μg/ml; and (■——■) berberine, 5 μg/ml.

Table 1. Effect of berberine incubated with S180 cells *in vitro* on various biochemical parameters*

Pathway	Per cent of control at 4 μg/ml	40 μg/ml
Acetate[^{14}C] → lipid	36.2	2.6
Glucose[^{14}C] → $^{14}CO_2$ (cells)	32.5	15.7
Glucose[^{14}C] → $^{14}CO_2$ (mitochondria†)	29.4	
Glycine[^{14}C] → protein	2.9	0.1
Thymidine[^{3}H] → DNA	48.9	1.6
Uridine[^{3}H] → RNA	13.4	0

* Details of incubation conditions are given in Materials and Methods.
† Mitochondrial suspensions were prepared from sonicated S180 cells by differential centrifugation at 4°.

DNA and protein respectively. The mean values for inhibition in three experiments were only 17 per cent for glycine and 14 per cent for thymidine uptake. On the other hand, when cells were harvested 30 min after treatment with berberine *in vivo*, washed, and then incubated with labeled glycine *in vitro*, incorporation of the amino acid into protein was reduced by 85 per cent as compared with cells removed from the same mice immediately before injection of drug. The percentage inhibition of glycine[^{14}C] incorporation by berberine *in vitro* was not affected by bringing the pH of the medium to 6 or by incubating under an atmosphere of helium.

Effect of glucose. When various concentrations of glucose were included in the incubation medium, the inhibitory effects of berberine on the incorporation of glycine[^{14}C] and thymidine[^{3}H] were partially or completely overcome (Table 2). The highest levels of glucose used are in the range reported for biological fluids [12]. this effect of glucose appears to originate from inhibition of berberine uptake. This is illustrated by the experiment depicted in Fig. 4. It is significant that the uptake of berberine is inhibited both by glucose and by phlorizin, an inhibitor of the active transport of glucose. As a corollary to this effect, we have also found that berberine can inhibit the uptake of glucose[^{14}C] by

Table 2. Effect of glucose on the inhibition of precursor incorporation in S180 cells by berberine *in vitro**

Glucose concn (mg/ml)	Per cent inhibition by berberine Protein synthesis	DNA synthesis
0	95.8	44.4
0.01	95.0	46.8
0.1	94.0	31.7
1.0	48.2	15.1
2.68	0	

* Berberine was added at 5 μg/ml. Cell suspensions were incubated in Krebs buffer with dialyzed calf serum, glucose and berberine, as indicated, for 30 min before addition of ^{14}C-labeled amino acid or tritium-labeled thymidine. Further treatment was as described in Materials and Methods.

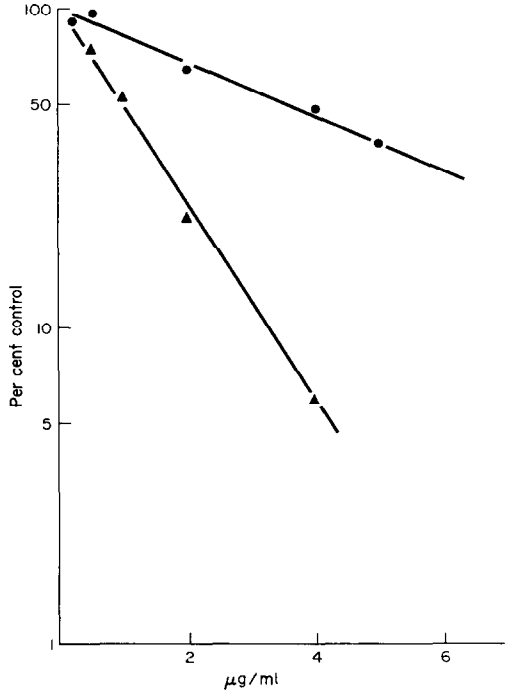


Fig. 3. Effect of berberine on the incorporation of thymidine[^{3}H] into DNA (●——●) and glycine[^{14}C] into protein (▲——▲) by S180 cells *in vitro*.

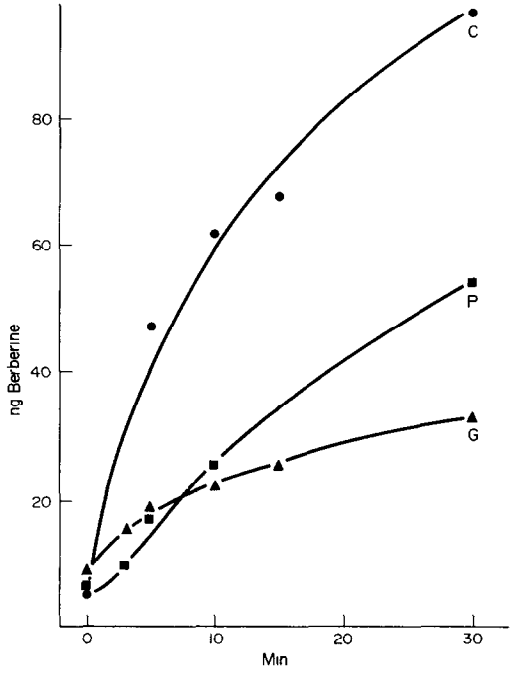


Fig. 4. Effect of glucose (1 mg/ml: ▲——▲) and phlorizin (1.4 mg/ml: ■——■) on the uptake of berberine (1 μg/ml: ●——●) by S180 cells *in vitro*. Drug was measured by fluorometric assay as described under Materials and Methods.

Table 3. Binding of berberine by polynucleotides in equilibrium dialysis experiments *

Polynucleotide	Amount of berberine bound (μg/mg polynucleotide)
Calf thymus DNA	54.0
Polyadenylic acid	163.7
Polycytidylic acid	0
Polyguanylic acid	90.7
Polyuridylic acid	0

* Polynucleotide (5 mg) was present inside a dialysis bag of volume 10 ml; the external volume was 100 ml. All experiments were carried out for 18 hr in the dark at room temperature. The system contained a total of 1 mg berberine.

S180 cells. Using the glucose[^{14}C] level and procedures described in Materials and Methods, berberine at 0.1 and 1.0 μg/ml inhibited glucose uptake by 45 and 82 per cent respectively.

Binding by polynucleotides. The mechanism involved in inhibition of nucleic acid biosynthesis was studied by examining the interaction of berberine with nucleic acid; both *d*-tetrandrine and thalicarpine interact with polynucleotides [7]. A hypochromic shift occurred in the spectrum of berberine (λ_{max} 347 nm) when DNA was added to the solution. This shift increased with the amount of DNA, and reached 25 per cent for 150 μg/ml of DNA mixed with 2 μg/mg of berberine. Using the method of equilibrium dialysis, association between berberine and DNA and other polynucleotides could be demonstrated (Table 3). Berberine showed the greatest affinity for polyadenylic acid, and did not appear to associate significantly with polycytidylic or polyuridylic acids.

DISCUSSION

There are definite resemblances as well as differences between the actions of berberine and of *d*-tetrandrine and thalicarpine which were studied previously [7]. All three agents inhibit the biosyntheses of DNA, RNA and protein by S180 cells *in vitro*. Since this inhibition becomes evident at concentrations below those that affect cellular uptake of precursors, it is likely that biosynthesis itself is being affected. Inhibitory activity *in vivo* is much more limited for thalicarpine and *d*-tetrandrine, and virtually nonexistent for berberine. Neither *d*-tetrandrine nor thalicarpine inhibits the oxidation of glucose to CO_2, which is a notable feature of berberine. In addition, very high concentration of these benzylisoquinoline alkaloids are required to affect the incorporation of acetate into lipids, again in contrast to berberine. However, in its inhibitory effect on glucose utilization, berberine does resemble papaverine which markedly inhibits respiration in C6 astrocytoma cells [13]. By virtue of its interaction with polynucleotides, berberine falls into the same class as *d*-tetrandrine and thalicarpine, except that it has no affinity for the pyrimidine bases, whereas thalicarpine is without affinity only for polycytidylic acid, and *d*-tetrandrine associates poorly with polythymidylic and not at all with polyuridylic acids [7].

The interaction with glucose is of interest, and probably underlies the ineffectiveness of berberine *in vivo*. It would appear from the evidence of mutual antagonism between berberine and glucose with respect to uptake as well as from the inhibition of berberine uptake by phlorizin, that the alkaloid is transported, in part, into cells by an active mechanism for glucose uptake, despite the discrepancy in chemical structure. Since the levels of glucose that significantly antagonize the inhibitory action of berberine on biosynthetic processes *in vitro* (Table 2) are in the range (~1 mg/ml) of natural glucose concentrations in the body fluid [12], it is not surprising that inhibitory effects are minimal *in vivo*, where drug levels will also be falling as metabolic and excretory clearance occurs. Even in culture *in vitro*, the inhibitory effect, although initially marked, is rather transient. Glucose levels in the final medium were about 0·8 mg/ml, not sufficient to antagonize entirely the inhibitory effect of berberine on biochemical pathways. The finding that inhibition of protein synthesis is evident in cells from drug-treated mice after they have been washed and incubated with glycine[^{14}C] *in vitro*, does not appear to be reconciled easily with the other data. It is possible that the presence of glucose in the external medium may facilitate the egress of berberine from the cells through some sort of cooperative interaction.

The relatively non-selective action of higher levels of berberine could reflect interference by the drug with glucose utilization. As energy-generating mechanisms are inhibited, all biochemical synthesis would be expected to cease. We have not yet explored the intracellular levels of nucleotides, such as ATP, which would provide a direct indication of the role played by inhibition of respiration.

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