Evidence of Active Transport of Benzyl-^{14}C-Penicillin from Cerebrospinal Fluid to Blood

ROBERT L. DIXON, ERNEST S. OWENS, and DAVID P. RALL

Abstract □ Data presented supports the hypothesis that benzylpenicillin is transported actively from the cerebrospinal fluid (CSF) to blood. Benzylpenicillin is capable of moving against a concentration gradient, undergoes self-saturation indicative of a transfer maximum, and is competitively inhibited by *p*-aminohippuric acid and probenecid. The locus of this transport mechanism appears to be the choroid plexus. It further appears that active movement of penicillin from CSF to blood may add to the difficulty of obtaining therapeutic concentrations of this antibiotic in the CNS. Data presented also support the concept that the cerebrospinal fluid functions as an escape channel for polar metabolites of neuronal metabolism and various polar drugs. Once a drug or metabolite enters the CSF from blood or brain by simple diffusion, it may be rapidly removed either by a nonselective bulk flow process *via* the arachnoid granulations or by an active transport mechanism.

Keyphrases □ Benzyl-^{14}C-penicillin transport—cerebrospinal fluid to blood □ Ventriculocisternal perfusion—drug administration □ Autoradiography—radioactivity determination □ Scintillometry—analysis

Failure of many chemotherapeutic agents to penetrate the central nervous system (CNS) in therapeutically effective quantities is a problem that has been recognized for many years. Abraham *et al.* (1) found penicillin in the blood, bile, and saliva, but not in cerebrospinal fluid (CSF). Many investigators have since confirmed these observations. Boger *et al.* (2) have summarized much of this work. Ullberg (3) studied the fate and distribution of ^{35}S-labeled benzylpenicillin using autoradiography and agreed with most of the observations made with microbiological assay methods which are summarized above.

Molecular size, pK, lipid solubility, and binding to proteins all contribute to the failure of many drugs to penetrate membrane systems such as the blood-brain and blood-CSF barriers (4). In addition to these factors, it now appears that an active mechanism might prevent the accumulation of certain drugs in the CSF. Various organic acids such as diodrast, phenolsulfonphthalein (PSP), and *p*-aminohippuric acid (PAH) have been shown to be transported actively from the CSF to blood (5, 6). The site of active transport is thought to be the choroidal epithelium. In this regard the choroid plexus resembles the renal proximal tubule, which is also capable of active secretion of various organic acids and bases (7). Data to be presented here indicate that benzylpenicillin, another organic acid actively secreted by the kidney, is also transported from CSF to blood by an active process. It appears possible that active movement of the antibiotic from CSF to blood adds to the difficulty of obtaining therapeutic concentrations of this drug in the CNS.

METHODS

Ventriculocisternal perfusions of dogs were performed in the following manner: mongrel dogs were lightly anesthetized with pentobarbital sodium, 30 mg./kg., administered intravenously (i.v.). The animals were placed in a sterotaxic apparatus[1] and the tip of a 7.62-cm. (3-in.), 22-gauge spinal needle was introduced into the right ventricle after making a burr hole in the skull. An outflow cannula consisted of polyethylene tubing attached to a 20-gauge needle which was placed percutaneously into the cisterna magna. The method was essentially the same as that described by Leusen (8) and modified by Pappenheimer *et al.* for goats (9). A portion of the ventricular system was then perfused with a synthetic CSF with ionic concentration and pH close to those found analytically (10).

The perfusate also contained benzyl-^{14}C-penicillin with a specific activity of 1.2 mc./mM[2] and inulin. Radiochemical purity of the benzyl-^{14}C-penicillin was found to be greater than 95%. This was determined using TLC (11). After chromatography, the penicillin was detected chemically using the iodine/azide reaction (11), and the radioactivity was estimated by autoradiography using X-ray film. The manufacturer stated the radiochemical purity to be >99% as determined by paper chromatography, chromatogram scanning, and autoradiography; chemical purity (>99%) was determined by IR absorption spectroscopy.

Radioactivity was analyzed using a spectrometer (Packard Tri-carb Liquid Scintillation). CSF, urine, plasma, and plasma ultrafiltrate were counted directly as 0.1–0.2-ml. aqueous samples in 18 ml. of 30% absolute methanol in toluene (v/v) with 3 g./l. of 2,5-diphenyloxazole (PPO) and 100 mg./l. of 1,4-bis-2(5-phenyloxazolyl)-benzene (POPOP) (12). Quenching was monitored by channel ratios. Inulin was determined by the resorcinol method corrected for CSF-inulin blank (13), or by double isotope counting using tritiated inulin.[3]

A constant infusion rate was obtained by the use of a calibrated infusion pump (Harvard), and outflow volumes were weighed periodically. Pressure in the ventricular system was regulated by the level of the cisternal outflow cannula. Experiments were performed at ventricular pressure of essentially zero pressure relative to the auditory meatus. This was accomplished by placing the tip of the outflow cannula at or below the level of the animal's auditory meatus. At zero or slightly negative pressure in the ventricle, the clearance of inulin from CSF, which is assumed to represent bulk absorption, was negligible (5, 14). Therefore, any disappearance of penicillin must be accounted for by diffusion and possibly active transport.

Transfer constants were calculated as described by Davson (15) and Pollay and Davson (16). The equation used was

$$\frac{dC}{dt} = k_{in}C_{pl} - k_f C_{out} - k_x C_{out} - k_{out}C$$

where k_{in} and k_{out} are transfer constants defining the rate of achievement of equilibrium or the steady state as a result of diffusional, *etc.*, forces operating across the blood-CSF barrier; k_f is the rate of secretion as a fraction of the total volume of the ventriculocisternal system, and k_x is the rate of perfusion expressed in the same way. The designs of these experiments were as follows: in one series, the perfusate contained benzyl-^{14}C-penicillin as previously described. In the second series, the perfusate contained no penicillin, and constant plasma levels were obtained after a single i.v. injection of benzyl-^{14}C-penicillin to dogs with both kidneys ligated.

[1] Model U, Baltimore Instrument Co., Inc.
[2] Atomic Accessories Inc.
[3] Inulin-T, 240 mc./g., Volk Radiochemical Co.

Plasma protein binding was determined by comparing the concentration of radioactivity (benzylpenicillin) in plasma to the concentration in a protein-free ultrafiltrate of the same sample. The ultrafiltrate was obtained by centrifuging a sample of plasma contained in an 20.32-cm. (8-in.) piece of cellulose dialyzing tubing.[4] The tubing was suspended in a 15-ml. centrifuge tube and secured by a rubber stopper during centrifugation. Filtrate (0.2–0.5 ml.) was obtained from a 2–3-ml. sample after centrifuging for 6 hr. at 750–1000 r.p.m. (125–225 × *g*) in a centrifuge (International PR-2) using a No. 269 head. The ultrafiltrate was determined to be free of protein. This method has been used successfully for the investigation of plasma protein binding of methotrexate (17), and was found to give results equivalent to the method described by Toribara *et al.* (18).

In vitro experiments of drug uptake by choroid plexus were performed as described by Welch (19) and Tochino and Schanker (20).

RESULTS

Figure 1 illustrates the design of a typical experiment. At zero time the perfusion was started and the cisternal outflow was collected every 20 or 30 min. The outflow volume and concentration of penicillin and inulin was determined and expressed as percent of inflow concentration. It can be seen that after 30–60 min., equilibration had taken place and the test substances in cisternal outflow remained relatively constant. In many instances, the perfusions were continued 4 or 5 hr. without significant change in cisternal outflow concentrations, suggesting that the disappearance of the antibiotic cannot be accounted for by drug tissue binding. At this rate of flow, 0.19 ml./min., the inulin concentration stabilized at about 80% of the inflow concentration. This was accounted for by dilution due to CSF production of about 0.05 ml./min., and represents a method used commonly to determine rate of CSF production (10). This rate of production for dogs, about 0.05 ml./min., was consistent with the findings of other investigators (10, 21). Benzyl-^{14}C-penicillin stabilized at about 60% of an inflow concentration of 1 mcg./ml. This figure can be accounted for by dilution due to CSF production and disappearance from the perfusate of approximately 0.05 mcg. of benzyl-^{14}C-penicillin per minute.

Table I summarizes clearance of benzylpenicillin from the CSF of dogs. The term clearance was used to represent the volume of CSF which was completely cleared of the test substance in 1 min. at a defined flow rate and drug concentration (5). In practice, clearance was determined from the steady-state difference between the quantity of drug entering the CNS and that leaving the brain each minute: quantity in − quantity out/mean CSF concentration. These data were obtained from experiments similar to that demonstrated in Fig. 1, in which the inflow rate was 0.19 ml./min. and the concentration of penicillin was 1 mcg./ml. Mean clearance of

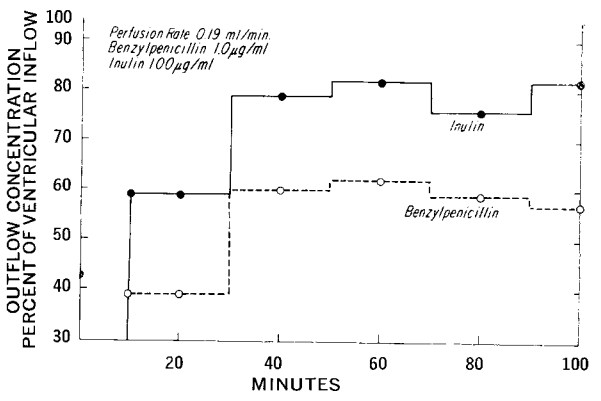

Figure 1—*CSF outflow concentrations of inulin and penicillin. Ventriculocisternal perfusion, at a rate of 0.19 ml./min., of a buffer containing 1.0 mcg./ml. of benzyl-^{14}C-penicillin and 100 mcg./ml. of inulin.*

[4] Union Carbide Corp. 0.991 cm. (0.390 in.) wide × 0.005 cm. (0.002 in.) thick.

Table I—Steady-State Clearance of Penicillin from CSF Perfusion

	Clearance,[a] ml./min.
Benzylpenicillin	0.13 ± 0.04 (9)[b]
Inulin	None

[a] Clearance = mcg. of drug in − mcg. of drug out/min./mean concentration of drug in CSF. [b] Mean ± standard error of nine experiments. All experiments had inflow concentration of 1 mcg./ml. and inflow rate of 0.19 ml./min.

penicillin for nine experiments was 0.13 ± 0.04 ml./min. The standard error indicates that these clearance figures are somewhat variable. Significant radioactivity (benzyl penicillin) could be detected in the urine of test animals at the end of the experiment. Clearance of inulin in these experiments was essentially zero because the experiments were performed at a pressure below that necessary for bulk flow. Only trace amounts of inulin could be detected in urine. These clearance values are only meaningful when stated along with perfusion rate and drug concentration, since changes in either parameter result in different values.

Clearance of benzyl-^{14}C-penicillin from the CSF was not significantly affected by blood concentrations of unlabeled benzylpenicillin which were calculated to be greater than 50 times higher than ventricular concentrations. Dogs received 25–50 mg./kg. of unlabeled benzylpenicillin administered i.v. as a single, slow injection during perfusion experiments similar to that represented by Fig. 1. This treatment had no effect on the percent of radioactivity appearing in the outflow. It was, therefore, assumed that penicillin clearance could take place against a concentration gradient.

The disappearance of benzyl-^{14}C-penicillin from CSF in these experiments cannot be accounted for by bulk flow. It also does not appear likely that passive diffusion of the drug or binding could completely account for the total amount of drug removed from the perfusate. Therefore, these data suggest that active transport may be involved. The following experiments were designed to further distinguish between active and passive processes.

Figure 2 shows the effect of saturation on the active transport mechanism for penicillin, one of six similar experiments. During the control period, the perfusion reservoir contained only trace quantities (0.25 mcg./ml.) of radioactive benzyl-^{14}C-penicillin. During this period, it can be seen that 30% of the inflow concentration of the drug was recovered. However, following the addition of 500 mcg./ml. of unlabeled benzylpenicillin to the reservoir, greater than 60% of the inflow concentration was recovered. At this flow rate, 0.08 ml./min., a dilution to 60% can be accounted for the CSF production of approximately 0.05 ml./min. Therefore, upon saturation, the percent radioactivity in the outflow is similar to that which is obtained with inulin. These data can also be expressed as extracted ratios $E = 1 - C_{out}/C_{in} \times$ outflow/inflow (5). During the control period, the extraction ratio for Fig. 2 was 0.50 and after saturation, the extraction ratio was zero. The extraction

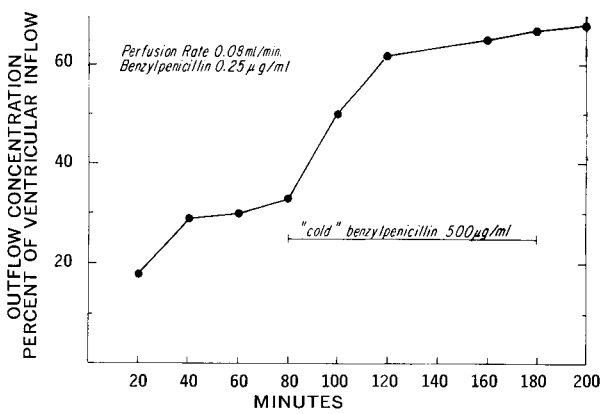

Figure 2—*Substrate saturation of penicillin transport. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of 500 mcg./ml. of unlabeled benzylpenicillin to the perfusate.*

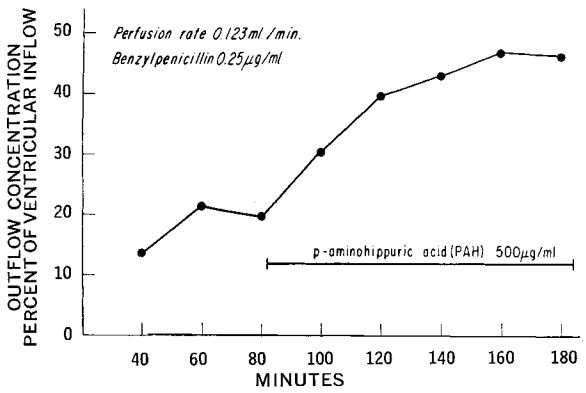

Figure 3—*Competitive inhibition of penicillin transport. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of PAH to the perfusate.*

ratio for inulin throughout these experiments was zero. Attempts to determine the transfer maximum (Tm) of penicillin were not successful due to the high variation between animals. However, data obtained indicated that the Tm for this system was low. It was of interest and should be noted that dogs under pentobarbital anesthesia exhibited signs of CNS stimulation (tremors and rigidity) immediately upon the addition to the perfusate of these rather high levels of penicillin.

Figures 3 and 4 demonstrate inhibition of penicillin transport by *p*-aminohippuric acid (PAH) and probenecid (Benemid). Again, trace quantities of benzyl-^{14}C-penicillin were perfused through the ventricular system until equilibrium was obtained. At this time, PAH or probenecid, 200–500 mcg./ml., was added to the reservoir. It can be seen that the extraction of penicillin from the perfusate was descreased upon the addition of PAH or probenecid. These data are in contrast to the addition of 500 mcg./ml. of *N*-methylnicotinamide chloride which had no effect on the extraction of penicillin. These experiments were suggestive of competitive inhibition of an active mechanism. Only a very small percent of PAH is bound to plasma protein and, therefore, it was unlikely that PAH was acting by a displacement of benzylpenicillin from CNS binding sites. Data from this laboratory indicated that PAH in concentrations 100 times that of benzyl-^{14}C-penicillin *in vitro* did not displace bound penicillin from 3.5% serum albumin or 5% brain homogenate in phosphate buffered saline. Also, CNS binding of benzyl-^{14}C-penicillin was not suggested by a constant cisternal outflow concentration over periods up to 5 hr. It must be pointed out, however, that the figures presented represent the majority of experiments, at least 10, where inhibition of benzyl penicillin transport was studied. However, in two of the experiments, no inhibition of benzyl-^{14}C-penicillin transport was demonstrated.

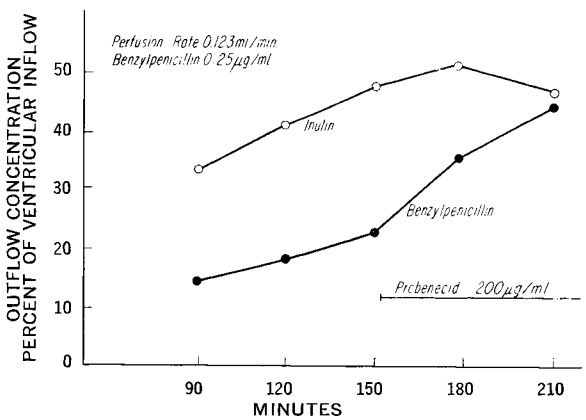

Figure 4—*Inhibition of penicillin transport by probenecid. Ventriculocisternal perfusion of buffer containing tracer quantities of benzyl-^{14}C-penicillin during control period followed by the addition of probenecid to the perfusate.*

Inward and outward transfer constants for benzylpenicillin were calculated. The rates of passage of benzylpenicillin across the membranes separating blood and ventricular compartments may be represented by transfer constants k_{in} and k_{out} as described by Davson (15). If the passage from CSF to blood is active, k_{in}, which is a measure of the ease of passage from blood to CSF, must be less than k_{out}. When a steady concentration of benzylpenicillin was maintained in the blood (renal ligation) during the course of ventriculocisternal perfusion with buffer containing no benzylpenicillin, a steady-state was established with a plasma/CSF ratio mean of 16 for ultrafiltrate and 25 for total plasma (Fig. 5). It can also be seen that 40–50% of benzylpenicillin was bound to plasma protein. The average values for the ratio of inflow concentration/outflow concentration of the experiments in which benzylpenicillin was in the perfusate using the same inflow rate, with essentially no drug in the blood, was approximately 1.6. From these two experimentally determined quantities, and from the known rate of perfusion (0.19 ml./min.) and the computed rate of secretion (0.05 ml./min.), it was possible to deduce that the ratio k_{out}/k_{in} was approximately five, assuming a ventricular volume of 3.0 ml. (22). That is, the passage from CSF to blood was some five times more rapid than in the reverse direction. Sample calculations have been presented by Pollay and Davson (16).

Table II presents data from *in vitro* experiments. These experiments are similar to those described by Welch (19). After rendering guinea pigs unconscious by cervical dislocation, individual choroid plexus and small pieces of ependyma-cortex were removed and placed immediately in oxygenated Tyrodes solution containing 1 mcg./ml. of benzyl-^{14}C-penicillin. After 45 min. at room temperature, the tissues were removed, drained, placed in weighed counting vials, digested in 0.1 ml. KOH, and the amount of radioactivity was determined. These data were expressed as a ratio of counts per milligram tissue divided by counts per microliter Tyrodes solution. It can be seen that the choroid plexus was able to concentrate the antibiotic nearly fourfold over the media in 45 min. This was in contrast to the ependyma-cortex which was much less able to concentrate the drug. Choroid plexus obtained by sacrificing three dogs revealed similar results. It was also demonstrated that the choroid plexus from either the lateral or fourth ventricle was capable of concentrating benzyl penicillin approximately fourfold over media. These data are similar to those reported by Tochino and Schanker (20) for quaternary ammonium compounds, and Rubin and Rall for methotrexate (23).

DISCUSSION

Data presented suggest that penicillin is transported actively from cerebrospinal fluid to blood. Experiments have been performed which indicate benzylpenicillin is capable of moving against

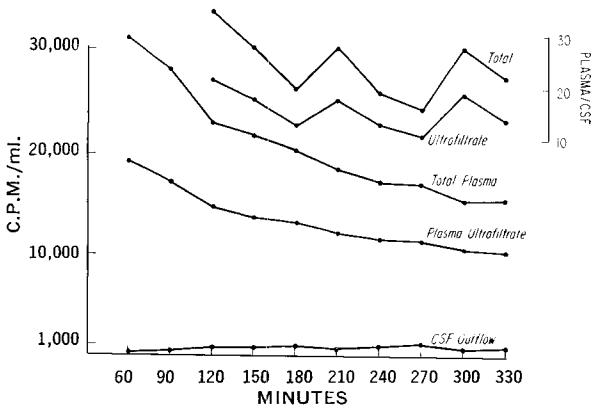

Figure 5—*Plasma and CSF concentrations of penicillin. Radioactivity (benzylpenicillin) in plasma, plasma ultrafiltrate, and CSF after the i.v. administration of 10 mg./kg. of unlabeled benzylpenicillin and 75 μc. of benzyl-^{14}C-penicillin to a 10 kg. dog following renal ligation. The ventricular system was perfused with plain buffer at a rate of 0.19 ml./min., during the entire experiment. One microgram of benzylpenicillin is equal to approximately 1200 c.p.m. The ratio of total plasma and plasma ultrafiltrate radioactivity to CSF radioactivity is also presented.*

Table II—Choroid Plexus Tissue/Medium Ratio[a] for Benzylpenicillin

Choroid plexus from lateral ventricle	3.8 ± 1.3 (18)[b]
Ependyma cortex	1.5 ± 0.8 (4)

[a] T/M = counts/mg. of wet tissue/counts/γl. of medium. [b] Each value is mean ± standard deviation. Figures in parentheses are number of determinations.

a concentration gradient, undergoes self-saturation indicative of a transfer maximum, and is competitively inhibited by PAH and probenecid. The locus of this transport mechanism appears to be the choroid plexus.

These results indicate that penicillin belongs to the class of organic acids that, like iodopyracet,[5] PAH, and PSP, are secreted by the proximal tubules of the kidney and transported from CSF to blood (6).

It appears possible that active movement of penicillin from CSF to blood may add to the difficulty of obtaining therapeutic concentrations of the antibiotic in the CNS. This possibility has been suggested previously by Pappenheimer *et al.* (5).

During the preparation of this paper, Fishman (24) published data concerning the blood-brain and CSF barriers to penicillin and related organic acids. In these studies, the disappearance rate of penicillin from the cisternal fluid was determined after a single injection of fluid containing penicillin and inulin. Many of the findings reported in the above research confirm a preliminary report (25), and agree with these data presented in this more extensive report, using the continuous ventricular-cisternal perfusion technique. Fishman also demonstrated that the rate of disappearance of penicillin from the CSF could be decreased by increasing the concentration of cisternal penicillin, or adding *p*-aminohippuric acid or probenecid to the CSF. In contrast, the disappearance of penicillin was not affected by the presence of high levels of penicillin in the systemic circulation after parenteral administration.

A number of factors have been shown to be responsible for excluding certain compounds from the CNS (4). These include the amount and lipid solubility of the unionized form of the drug at physiological pH. The amount of drug free from association with various binding sites, including plasma proteins, also determines the amount of drug available for membrane penetration. Benzylpenicillin has a relatively low molecular weight of 372, which would not be expected to be a major factor limiting its entry to the CNS.

However, benzylpenicillin is a weak organic acid with a pKa of 2.6, which therefore is highly ionized at pH 7.4. Thus the slow diffusion of penicillin across the blood-CSF membranes reflects the poor lipid solubility of ionized forms of drugs. With a pKa of 2.6, the normal pH difference between blood (7.40) and CSF (7.32) results in only a small difference in total drug concentration on each side of the membrane. There is also an equilibrium in blood between protein-bound and protein-free penicillin. Benzylpenicillin is bound about 50% at concentrations near the therapeutic level (26) and it is only the unbound or free form that is available for membrane penetration. Thus, the chemical characteristics of penicillin are major factors limiting its entry into the brain and CSF (24).

Data presented here suggest that an active movement of drugs from CSF to blood may also contribute to the failure of many drugs, like those actively secreted by the renal proximal tubule, to accumulate in the CNS. These data support the concept that the cerebrospinal fluid functions as an escape channel for polar metabolites of neuronal metabolism and various polar drugs (27). It appears that once a drug or metabolite enters the CSF from blood or brain by simple diffusion, it may be rapidly removed, either by a nonselective bulk flow *via* the arachnoid granulations (28), or by an active transport mechanism.

[5] Diodrast, Winthrop Laboratories, New York, N. Y.

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