Penetration of Sugars, Steroids, Amino Acids, and Other Organic Compounds into the Interior of the Squid Giant Axon

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ABSTRACT Squid axons were exposed to solutions of C^{14}-labeled compounds. After 60 minutes the axoplasm was extruded and assayed for radioactivity. The following compounds penetrated to about 3 per cent of what would have been expected had there been no barrier to free diffusion and the subsequent attainment of equivalent distribution: mannitol, sucrose, glutamate, glutamine, aspartate, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, γ-aminobutyrate, serotonin, and dehydroepiandrosterone sulfate. All these compounds are water-soluble; in addition, some are ionized over the entire pH range. Partially ionized indoleacetate, acetylsalicylate, and 5,5-diphenylhydantoin penetrated about 40 per cent and unionized, water-insoluble cortisol and dieldrin, 100 per cent. A striking exception to this grouping was glucose, which penetrated about 20 per cent. Studies with specifically labeled glucose indicate participation of the pentose phosphate pathway as a metabolic route in axonal membrane and associated cell wall material, and partial or complete absence of the oxidative system in the axoplasm. Except for glucose, penetration of the substances studied appears to depend largely on the extent of the non-polar, lipophilic character of the compound. Penetration can be markedly increased by pretreatment of the axons with cottonmouth moccasin venom.

INTRODUCTION

Substances interacting with biological systems must do so at or within the cell membrane, or must penetrate the membrane in order to interact with intracellular components. The penetration into the squid giant axon of compounds related to the acetylcholine system has been examined (1, 2), as well as the inhibition or facilitation of such penetration afforded by some related compounds (3). We have now examined the penetration into the squid axon of many complex organic molecules including sugars, steroids, and amino acids, of which some are known to have a special function in the nervous

system. It is probable that the metabolism of glucose mediates its entry into cells, so the metabolism by the squid axon of C^{14}-labeled glucose to $C^{14}O_2$ has also been examined. The squid axon is suitable for studies on the permeability of the nerve plasma membrane: it is a single axon, although surrounded by a Schwann cell; the condition of the membrane can be monitored throughout the experiment in terms of its action potential; the axoplasm can be extruded and assayed without consideration of diffusion into extracellular spaces or of non-specific adsorption.

MATERIALS AND METHODS

Descriptions have previously been given (2, 3) of the preparation of the giant axon of the squid (*Loligo pealii*), of the recording of electrical activity, of the application of cottonmouth moccasin venom (*Agkistrodon p. piscivorus*), of the extrusion of axoplasm, and of the measurement of radioactivity and the arithmetical treatment of data.

For the metabolism of glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}O_2$ the methods were essentially those reported previously for other tissues (4). Sea water from Woods Hole was boiled for 15 minutes, cooled, restored to original volume by the addition of distilled water, filtered, and oxygenated prior to use as incubation medium. During oxygenation, pH was adjusted to between 7.2 and 7.4 by addition of HCl. The metabolism of whole axons, axoplasm-free axons, and axoplasm alone was measured in terms of O_2 utilized and $C^{14}O_2$ released. Cleanly dissected axons, freed of all small fibers, were blotted. The ligatures at both ends were cut off, and the axons were quickly weighed and transferred into ice cold, previously prepared Warburg vessels. To obtain membrane free of axoplasm, a cleanly dissected axon was placed on a glass slide submerged in cold sea water; the ligature at one end was cut off and the axon was rolled with considerable force with a tygon-coated roller. After the axon was thoroughly flattened, the ligature was removed from the other end; several such axons were collected, weighed, and transferred into the Warburg vessel. To obtain axoplasm alone, several crudely dissected axons were extruded in the usual manner (2) onto a weighed cover glass, and the cover glass with axoplasm was dropped into the Warburg vessel. Incubation of such systems with glucose-1-C^{14} or glucose-6-C^{14} lasted for 3 hours at 30°C. The contents of the center wells were then assayed for radioactivity.

For the penetration studies compounds labeled with C^{14} were dissolved in sea water made 0.001 M in tris(hydroxymethyl)aminomethane (pH 7.8). When necessary the pH was readjusted after dissolution of the compound. Dieldrin,[1] an extremely water-insoluble compound, was taken up in absolute ethyl alcohol and dispersed into aqueous medium by adding 0.10 ml of the hot alcoholic solution to 100 ml of ice cold, buffered sea water which was stirred violently. The resulting colloidal suspension appeared

[1] The following trivial names and abbreviations are used: dieldrin, 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene; ACh, acetylcholine; DPN, DPNH, TPN, TPNH, oxidized and reduced di- and triphosphopyridine nucleotide; GABA, γ-aminobutyric acid; dopa, DL-3,4-dihydroxyphenylalanine; dopamine, 3,4-dihydroxyphenylethylamine; serotonin, 5-hydroxytryptamine; aspirin, acetylsalicylic acid; dilantin, 5,5-diphenylhydantoin; U (applied to radioactive compounds) uniformly labeled.

clear in transmitted light, showed a Tyndall effect when viewed in right angle incident light, and showed no evidence of settling during 48 hours. This concentration of the alcohol had no effect on axonal function (5).

Squid axons were immersed in the test solutions for 60 minutes at about 20°C prior to analysis of the axoplasm for uptake activity (2).

TABLE I

PENETRATION OF SUGARS INTO AXOPLASM OF SQUID AXON

The concentration of C^{14}-sugars was 4.5×10^{-3} M in all experiments. Venom-treated axons were exposed to venom until block of electrical activity (30 to 45 minutes) and then washed for 10 minutes. Exposure to radioactive solution was 60 minutes in all experiments. The expected values are those which would be found in the axoplasm if there were no barrier to penetration and equivalent distribution were attained.

Compound	Venom concentration	Disintegrations/min./sample		Penetration
		Expected	Found	
	μg/ml			*per cent*
Glucose-1-C^{14}	0	731	170	23.3
	0	1713	322	18.8
Glucose-6-C^{14}	0	694	158	22.8
	0	1711	348	20.3
Mannitol-1-C^{14}	0	2812	17	0.60
	0	2979	34	1.14
Sucrose-U-C^{14}	0	452	12	2.65
	0	694	2	0.29
	100	400	143	35.8
	100	285	118	41.4

Dieldrin-1,2,3,4,10-C^{14} was obtained as a gift from Shell Chemical Company, dehydroepiandrosterone sulfate-4-C^{14} as a gift from Dr. Erlio Gurpide of this University. Other radioactive compounds were obtained commercially. Lyophilized cottonmouth venom (lot 7/9/63) was purchased from Ross Allen Reptile Institute, Silver Springs, Florida.

RESULTS AND DISCUSSION

Table I shows the penetration of glucose into the squid axon, and the lack of penetration of mannitol and sucrose. The results with sucrose are in agreement with previous findings on finely dissected squid axons (6). Pretreatment of the axons with cottonmouth moccasin venom permitted the penetration of sucrose reminiscent of the results with ACh (2). It appears likely that the glucose penetration is metabolically mediated.

The production of $C^{14}O_2$ from glucose-1-C^{14} and from glucose-6-C^{14} by

squid axons is shown in Table II. The greater production of $C^{14}O_2$ from glucose-1-C^{14} than from glucose-6-C^{14} indicates some participation of a pentose phosphate pathway, although the extent of participation cannot be accurately estimated without at least a knowledge of the extent of the other possible pathways of glucose metabolism (7). An even greater difference between CO_2 production from carbon 1 and from carbon 6 has been found in another highly

TABLE II

METABOLISM OF SPECIFICALLY LABELED GLUCOSE BY SQUID AXONAL MATERIAL

Each vessel contained, in the main compartment, 15 μmoles glucose (radioactive + non-radioactive), tissue, sufficient treated sea water (see Materials and Methods) to give an incubation volume of 1.5 ml; in side arm, 0.15 ml 5 N H_2SO_4; in center well, 10 per cent KOH plus filter paper wick; O_2 atmosphere; temperature, 30°. Incubation started 5 minutes after vessel was placed in Warburg bath, terminated 3 hours later by tipping side arm contents into main compartment. All results expressed per gram fresh material and 3 hours. Actual weights used, on the average, 26 mg of whole axon (4 axons), 3 mg of axoplasm-free axon (4 axons), and 13 mg axoplasm. Vessel constants, 0.5–0.6.

Tissue	Radiochemical yield of $C^{14}O_2$ from G-1-C^{14}	Radiochemical yield of $C^{14}O_2$ from G-6-C^{14}	Ratio $\frac{\text{G-6-C}^{14}}{\text{G-1-C}^{14}}$	O_2 uptake ± standard deviation
	per cent	*per cent*		*mm³*
Whole axon	1.46	0.336	0.23	330±26
	1.48	0.356	0.24	207±42
Axoplasm-free axon	2.63	0.703	0.27	—*
	3.09	0.894	0.29	—*
Axoplasm	0	0		—*
	0	0		—*

* See footnote 2 in text.

specialized nerve tissue, the electric tissue of *Electrophorus electricus* (4). It has in this case been assumed that pentose phosphate pathway participation is negligible (8) because TPN + TPNH is present in this tissue at only about 5 per cent of the DPN + DPNH level. This does not disprove the possibility of the pentose pathway, however, since an extremely small amount of an endogenous easily reducible and re-oxidizable compound such as coenzyme Q could permit even such a small amount of TPN + TPNH to participate in the operation of the pathway (9).

The ability of the perfused squid giant axon to conduct about 3×10^5 impulses has led to the conclusion (10) that, if Nachmansohn's theory of nerve conduction (11) is correct, the reactants and catalysts of energy pro-

duction must be assumed to be rather tightly bound to the axonal membrane. Our results (Table II) indicate that at least some of the oxidative enzymes and cofactors are indeed bound to the membrane and cell wall material.

TABLE III

PENETRATION OF AMINO ACIDS INTO AXOPLASM OF SQUID AXON

The concentration of C^{14}-amino acids was 4.5×10^{-3} M in all experiments. Venom-treated axons were exposed to 25 μg/ml venom for 30 minutes, or to 100 μg/ml venom until block of electrical activity (30 to 45 minutes) and then washed for 10 minutes. Otherwise, see Table I.

Compound	Venom concentration	Disintegrations/min./sample		Penetration
		Expected	Found	
	μg/ml			*per cent*
L-Glutamate-U-C^{14}	0	1322	23	1.74
	25	1583	112	7.08
	100	2624	302	11.5
	100	1700	771	45.4
	100	4765	1783	37.4
L-Glutamine-U-C^{14}	0	1718	62	3.61
	0	3080	23	0.75
	100	742	132	17.8
	100	3685	2652	72.0
	100	2658	804	30.2
	100	2425	440	18.1
L-Aspartate-U-C^{14}	0	3075	57	1.85
	0	3150	66	2.10
	0	3135	81	2.58
GABA-1-C^{14}	0	5650	220	3.89
	0	2480	97	3.91
	0	2940	82	2.79
	25	2187	360	16.5
	25	3205	223	6.96
	25	1997	103	5.16
	100	2418	537	22.2
	100	2838	681	24.0

Since the axoplasm constituted about 90 per cent of the weight of our cleanly dissected axons, and since the axoplasm does not produce *any* CO_2 from added glucose (Table II), the production by the axoplasm-free preparation might have been expected to be ten times that of the whole axon on a weight basis, provided that the oxidative system was completely undamaged. In fact, there was a twofold increase, but the oxygen uptake, never large, was no longer measurable. The tenfold increase of $C^{14}O_2$ production expected and

the twofold increase found represent, therefore, an apparent decrease (*i.e.*, one-fifth as large an increase as was expected). This decrease in $C^{14}O_2$ production and the apparent loss of oxygen uptake[2] may result from damage to the oxidative system during the rolling and crushing of the axon in the extrusion of the axoplasm.

Table III shows that glutamate, glutamine, GABA, and aspartate penetrated the untreated squid axon to less than 4 per cent of what would have been expected due to free diffusion in the 60 minutes allowed for penetration studies. Dopa also appeared to be subject to a marked permeability barrier (Table IV). This probably reflected the fact that all five compounds exist almost always in a charged form. This low penetration was in marked contrast to the uptake of the first three by brain slices (12) but is in fair agreement with Korey's finding (13) that aspartate penetrates slowly into squid axons. A low concentration of cottonmouth venom, 25 μg/ml, increased penetration, and this was increased still further by a venom concentration (100 μg/ml) which of itself destroys axonal conduction.

The two amines, dopamine and serotonin, also have two functional groups. However, both the $-NH_3^+$ and the phenolic —OH have pK_a's of 9 to 10; because of this closeness of the pK_a's there will be a somewhat greater probability of the molecules existing, at any given time, in the uncharged form. Table IV shows that dopamine and serotonin, although almost excluded by the intact axonal membrane, could penetrate the venom-treated axon, on the average, to a somewhat greater extent than the amino acids. The assumption has been made (14) by extrapolation from studies of the penetration of other catecholamines through the blood-brain barrier that dopa would probably penetrate membranes, but not dopamine. The uptake of dopa and other amino acids by brain slices (15) may be more a function of brain metabolism than of membrane permeability.

The compounds under consideration in the two previous paragraphs have two or more ionizable groups each, selected from carboxyl, amine, and phenolic OH. Aspirin, indoleacetic acid, and dilantin have only one each. Table IV shows that these compounds penetrate the untreated squid axon much more rapidly. Even at the pH used in the bathing solution these compounds will be in equilibrium with a significant amount of the undissociated, lipophilic form. In fact, dilantin is largely undissociated, poorly water-soluble, and penetrates to a greater extent than the other two at a rate in good agreement with values found previously under similar conditions (16). Our results with aspirin and indoleacetic acid are essentially in agreement with those on

[2] In each experiment 20 to 30 mg of whole axon caused a 10 to 20 mm change in manometer fluid height in 3 hours. The same number of axons freed of axoplasm weighed 2 to 3 mg. If, in these axoplasm-free axons, there were an oxygen uptake equivalent to the relatively decreased $C^{14}O_2$ production, the resultant change of 2 to 4 mm would have been immeasurably small.

the absorption of aspirin and other arylcarboxylic acids from the stomach (17). Although it is claimed by Schanker *et al.* (17) that the absorption of such acidic drugs is impeded in an alkaline gastric medium, the published results

TABLE IV

PENETRATION OF SOME AROMATIC COMPOUNDS INTO AXOPLASM OF SQUID AXON

The concentration of indoleacetic acid was 1×10^{-3} M; dilantin, 2.5×10^{-4} M; all others, 4.5×10^{-3} M. Otherwise see Table III.

Compounds	Venom concentration	Disintegrations/min./sample		Penetration
		Expected	Found	
	μg/ml			*per cent*
Dopa-carboxyl-C^{14}	0	2580	62	2.40
	0	4407	70	1.59
	0	3235	12	0.37
	100	514	45	8.75
	100	2000	712	35.6
	100	2820	1902	67.4
Dopamine-ω-C^{14}	0	2577	100	3.88
	0	1675	41	2.45
	100	2078	1292	62.2
	100	534	415	77.7
Serotonin-2-C^{14}	0	3340	168	5.03
(side chain label)	0	4025	206	5.12
	0	4215	338	8.02
	100	3716	1854	49.9
	100	3474	2000	57.6
Indoleacetic acid-2-C^{14}	0	3135	790	25.2
(side chain label)	0	508	57	11.2
	0	3945	1172	29.7
Aspirin-carboxyl-C^{14}	0	3034	780	25.7
	0	3180	1493	46.9
Dilantin-4-C^{14}	0	6350	3733	58.8
	0	4920	2772	56.3
	0	3290	2654	80.7

indicate that the impedance is not directly proportional to [Ar COO^-] or even to p[Ar COO^-].

Table V shows the results obtained using three compounds of rather diverse biological interest, and of equally diverse physical properties. Cortisol is a lipid-soluble steroid but, for a steroid, quite water-soluble; dehydroepiandrosterone sulfate is a water-soluble, completely ionized derivative of an

otherwise water-insoluble steroid; dieldrin is a highly toxic, extremely water-insoluble insecticide. Both cortisol and dieldrin penetrate fully into the axoplasm of the squid axon. In marked contrast, dehydroepiandrosterone sulfate is almost completely excluded. Even the 4 per cent penetration shown in Table V might really be the penetration of dehydroepiandrosterone itself formed by the action of hydrolytic enzymes on the sulfate ester. With respect

TABLE V

PENETRATION OF A STEROL, A STEROL SULFATE, AND A CHLORINATED HYDROCARBON INSECTICIDE INTO AXOPLASM OF SQUID AXON

The concentration of cortisol and dehydroepiandrosterone sulfate was 2.5×10^{-4} M; of dieldrin, 1.9×10^{-6} M (assuming it was in solution). Venom was not used. Otherwise see Table I.

Compound	Disintegrations/min./sample		Penetration
	Expected	Found	
			per cent
Cortisol-4-C^{14}	298	280	94.0
	276	268	97.1
	1148	1071	93.3
	735	933	126.9
Dehydroepiandrosterone sulfate-4-C^{14}	565	23	4.07
	370	16	4.32
	321	12	3.74
Dieldrin-1,2,3,4,10-C^{14}*	291	126	43.3
	401	445	111.0
	482	486	100.8
	473	620	131.1

* See Metcalf (18) for numbering.

to cortisol and dieldrin penetration, the present results do not indicate whether equilibrium has been attained or whether there may be a large accumulation in the cell membrane and associated cell wall material. Dieldrin and the chlorinated hydrocarbon insecticides in general penetrate tissue readily and are accumulated in toxic form in lipid depots (18). The steroid and the steroid sulfate thus constitute a striking example of the effect on permeability of introducing a highly polar, completely dissociated substituent into a lipophilic molecule. Similar results have been demonstrated using the perfused placenta (19).

No effect was visible on the propagated action potential, nor on its threshold using the experimental compounds at the concentrations and for the times of

application employed. The measurement of electrical properties served to indicate the intactness of the axonal membrane.

The passage of compounds through cell membranes and associated structures into cells depends partly on their relationship to an uncharged, nonpolar, lipid-soluble state. The penetration of many compounds can be increased by the action of venoms, probably as a result of the action of phospholipase A (20) on the permeability barriers. These conclusions are based on experiments with squid axons but it appears possible that cell membranes from widely different sources may have sufficient properties in common to give some validity to such generalizations.

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