Kotani *et al*(17) and confirmed in this work, but afford poor protection in the pulmonary challenge test(1,10). The effect of oil is not yet understood. Work in progress has shown that removal of most of the oil from an oil disruption product by extracting it with petroleum ether in a Soxhlet apparatus did not impair the resistance-enhancing potency or restore allergenicity.

Summary. The protective values against experimental tuberculosis in mice of Youmans' cytoplasmic particulate fraction and of oil disruption product, both prepared from the same lots of *Mycobacterium tuberculosis,* strains BCG and H37Ra, were tested in parallel with viable BCG vaccine. Two tests were employed, one based on intravenous challenge with large doses and the other on pulmonary challenge with a few virulent tubercle bacilli (H37Rv). Results are presented demonstrating that the vaccine efficacy of the oil disruption product approached or equalled that of similar doses of viable BCG by all criteria used to estimate the response, while its ability to sensitize guinea pigs to PPD was considerably less than that of viable BCG. The cytoplasmic particulate fraction prepared according to the method of Youmans *et al,* while effective at high dose levels in the massive challenge test, was not effective in the aerosol challenge test. In contrast to the particulate fraction, sterilization by heat did not impair the protective potency of oil disruption product.

The authors express gratitude to the Montana Tuberculosis Association for the technical services of Larry Ewalt.

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Received November 12, 1964. P.S.E.B.M., 1965, v118.

Intestinal Transport of Amino Acids and Glucose in Flounder Fish.* (30010)

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The transmucosal movement of aromatic amino acids and glucose by mammalian and amphibian intestine has been widely reported and is considered to be an active process. Little is known about the intestinal transport

* This study was supported by grants of Nat. Science Foundation (GB-2113) and Nat. Inst. of Arthritis & Metab. Dis., NIH, (AM-02217-07).
† Supported by U.S.P.H.S. training grant 5 T1 GM 461, Nat. Inst. of Gen. Med. Sci.

of these compounds in marine fishes. Morphologically, the intestine of marine fishes is similar to that of the other animals. However, the length of the fish intestine, depending upon their feeding habits, is relatively shorter than that of the mammals. The question arises, does the fish intestine function similarly to that of mammals, namely, having a specific mechanism to absorb the digested nutrients, such as glucose and amino acids? Our previous report showed the active transport of glucose across the mucosal membrane of dog fish(1). This paper describes our study on intestinal transport in flounder fish, a teleost, and shows that this transport is quite different in flounder than in mammalian intestine.

Experimental. Materials. The tyrosine and tryptophan derivatives used in this study were purchased from Nutritional Biochemicals Corp. (Cleveland), except for the compound 3,5-dichlorotyrosine which was synthesized in this laboratory(2). Radioactive L-tryptophan-3-C^{14} was obtained from the New England Nuclear Corp. (Mass.). Probenecid was obtained from the Merck Institute for Therapeutic Research through the courtesy of Dr. J. E. Baer. All compounds were dissolved in equimolar sodium hydroxide solution and diluted to the desired concentration with incubation solution.

Procedure. Winter flounders (*Pseudopleuronectes americanus*) were caught either by hook or by dragging in the gulf of Maine during the months of July and August. They were kept in a live cart for at least 2-4 days. When ready for use, they had usually recovered from the effects of capture and swam vigorously about the cart. The length of the fish varied from 8 to 15 inches. They were killed by hitting the head with a hammer blow. The abdomen was opened and the intestine was removed from about 2 cm down from the stomach to about 2 cm away from the rectum. The intestinal lumen was washed out with cold seawater, everted, and divided into 3 or 4 segments, approximately 2.0 cm in length. Details of the procedure have been described by Wiseman(3).

The incubation solution was made up of "Sea-water Ringer" with the test compound and was aerated with 5% CO_2-95% O_2 for 15 minutes. Five ml of this solution were pipetted into a container, and 1 ml of the same solution was placed into the everted sac. The flask was then gassed for 30 more seconds and closed with a stopper and incubated in a Dubnoff shaker at 25°C for an hour.

The composition of "Sea-water Ringer" was as follows: To one liter of sea water§ are added KCl 12 mmols. $CaCl_2$ 10 mmols, KH_2PO_4 0.5 mmols, $NaHCO_3$ 4.5 mmols, glucose 11 mmols and inulin 500 mg. The final osmolarity of the sea-water Ringer was 920 mOsm. When "Sucrose-Ringer" was used to substitute for "Sea-water Ringer," a 0.92 M sucrose solution was used instead of sea water, and the other components remained the same.

Analysis. The method of Folin-Ciocalteau was used to determine tyrosine and its analogues(4). The tryptophan derivatives were determined by the method of Spies and Chambers(5). Glucose was analyzed by glucose oxidase method or Nelson's method(6). Inulin was analyzed by the method of Roe *et al*(7). Inulin space was used to calculate the change of serosal fluid after incubation. This was based on the assumption that inulin had not penetrated through the serosal membrane. The change of the inulin concentration would indicate either the gain or the loss of serosal fluid. The net rate of transport of the compound was calculated from the difference between the final and original amount of the amino acid in serosal sacs ($= C_fV_f - C_o \times 1$, where V_f is the final volume of serosal fluid determined by inulin method).

The radioactivity of C^{14} labeled tryptophan compound in serosal and mucosal fluid was counted with a Nuclear Chicago gas flow planchet counter.

Results. Thirty-six flounders were used in this study. The fish can be divided into 2 groups; the big fish (over 12 inches in length) had a thick intestinal wall, and the smaller ones (below 12 inches in length) had a thinner wall. In all everted sacs studied here, the final inulin concentration in serosal

§ The chemical composition of Sea-water is given in Venogrados, A. P.: The elementary chemical composition of Marine Organisms. Memoir II. Translated from the Russian, Yale Univ., 1953, p14.

TABLE I. Intestinal Transport of Tyrosine and Its Analogues.

Compound	Initial conc (mM)	Final conc Mucosa	Final conc Serosa	Rate of transport (μmole/g/hr)
L-tyrosine	2.5	2.3	4.1	4.79 ± .72 (5)* [1.91 ± .45 (6)]†
D-tyrosine	4.2	3.96	5.1	5.31 ± 1.03 (2)
	1.3	1.09	2.0	5.55 (1) [2.0 ± .62 (5)]†
DL-*m*-tyrosine	2.25	1.86	3.2	4.68 ± .32 (3) [1.12 ± .05 (2)]†
DL-*o*-tyrosine	2.8	2.6	3.5	3.75 ± .035 (2) [1.46 ± .63 (3)]†
L-DOPA	2.0	1.85	4.55	7.1 ± 2.92 (5)
L-3-NH_2-tyrosine	2.0	1.85	2.95	1.01 ± .42 (2)
3,5-Dichloro-L-tyrosine	2.0	1.90	2.25	1.07 ± .01 (2)
3,5-Dibromo-tyrosine	2.0	2.27	1.81 (3)	—
3,5-DiNO_2-tyrosine	2.0	1.15	1.82 (2)	—

* Mean ± standard deviation. Numbers in parentheses are numbers of experiments.
† Sacs from thick intestine. The tissue weight was usually twice as heavy as the weight of thin intestine.

fluid was slightly increased, indicating a fluid movement from the serosal to tissue or to mucosal side. The sac of the thicker intestine lost more fluid than the intestinal sac with the thinner wall.

Transport of glucose. Two concentrations of glucose, 5.5 mM and 11 mM, were used in the everted sac method. No active transport of glucose was observed. The final concentration of serosal fluid was always below the original serosal concentration.

Transport of tyrosine and its analogues. While the glucose was not transported actively by the intestinal mucosa, tyrosine and its analogues were found to be transported in the same intestinal sac. Although the rate of transport from fish to fish varied, depending upon the thickness of intestinal wall, the rate of transport in different segments from one fish intestine varied insignificantly.

Table I summarizes the data on intestinal transport of tyrosine and its analogues. It was found that L-tyrosine, D-tyrosine, 3,4-dihydroxyphenylalanine (DOPA), meta-tyrosine, ortho-tyrosine, 3-NH_2, and 3,5-dichloro-tyrosine were transported from mucosal to serosal side against a concentration gradient. 3,5-dibromo and dinitro-tyrosine were not transported.

As shown in the Table the rate of amino acid transport by the thick intestine was smaller than that of the thinner intestinal sacs. Since rate of transport of these compounds was calculated per unit of wet intestinal weight, not per unit of area of mucosal surface, it is difficult to determine whether the thickness of the intestinal wall acts as a hindrance for the trans-mucosal movement, or the increase of weight without the consequent increase in transported area yields the misleading reduction of rate of transport.

Effect of sodium replacement on tyrosine transport. When sucrose Ringer was used to replace sea-water Ringer as incubation fluid, in both mucosal and serosal side, the rate of transport of L-tyrosine was partially inhibited. If the sucrose Ringer was replaced by the sea-water Ringer on the mucosal side only, the rate of transport of L-tyrosine was not affected; in fact it was slightly increased (Table II).

TABLE II. Effect of Sucrose-Ringer on Intestinal Transport of L-tyrosine.*

Replacement on:	% of control value:†
1. Both sides	67
2. Mucosal side	109
3. Serosal "	90

* 0.92 M sucrose replaced sea-water (see text). Concentration of L-tyrosine in both mucosal and serosal side was 2.4 mM.
† Avg of 2 exp.

TABLE III. Effect of Inhibitors on Intestinal Transport of L-tyrosine.

Inhibitor	Conc (mM)	Rate of transport (% of control value)*
L-phenylalanine	20	42.5 ± 4.6 (3)
Sodium azide	10	58.0 ± 12.2 (4)
2,4-dinitrophenol	.5	52.0 ± 24.5 (3)
Probenecid	5.0	34.0 ± 6.7 (3)

* To eliminate the variation of rate of transport between small and big flounders, the comparison was observed in the same fish intestine.

Effect of inhibitors on tyrosine transport. Table III summarizes the effects of 4 different inhibitors on intestinal transport of L-tyrosine. L-phenylalanine, sodium azide, 2,4-dinitrophenol, and probenecid all inhibited the transport of L-tyrosine.

Transport of tryptophan and its derivatives. The flounder intestine, similar to that of golden hamster, did transport L-tryptophan, but not D-tryptophan or the decarboxylated analogue, tryptamine (Table IV).

Flux movement of L-tryptophan across the intestinal wall. C^{14} labeled L-tryptophan was added into the non-labeled L-tryptophan incubation fluid to study the flux movement of L-tryptophan. Table V presents one of 3 experiments in this study. It can be seen here that C^{14}-labeled tryptophan was moved in the same direction as the non-labeled compound from mucosal side to serosal side. When the labeled compound was placed only in serosal fluid, very little radioactivity was detected in the mucosal fluid after an hour's incubation.

Discussion. Data presented here demonstrate two facts which are significantly different from that observed in mammalian and amphibian intestines.

(a) Flounder intestines do not transport glucose across the mucosa but do transport tyrosine and tryptophan against a concentration gradient. Previous investigation has shown that glucose was transported across a mucosal membrane of dogfish against a concentration gradient, but was not moved across an intact intestinal wall actively(1). This suggests that a barrier exists in the muscular and serosal layers which prevents the movement of glucose from the mucosal to serosal side. Preparation to separate the mucosal membrane from flounder intestine for trans-

TABLE IV. Intestinal Transport of Tryptophan Derivatives.

Compound	Initial conc (mM)	Final concentration		Rate of transport (μM/g wet wt)
		Mucosal	Serosal	
L-tryptophan	2.0	1.4–1.82	2.22–3.12	2.82 ± .05 (2)* [.9 ± .37 (9)]†
D-tryptophan	2.0	1.85	1.65 (4)	—
Tryptamine	2.0	1.45	1.50 (3)	—

* Mean ± standard deviation, numbers in parentheses are numbers of experiments.
† Sacs from thick intestine.

TABLE V. Intestinal Transport of L-tryptophan and L-tryptophan-3-C^{14}.*

Exp No.		Mucosal fluid		Serosal fluid		Intestinal tissue†	
		C^{14} conc 10^5 c/ml (min)	SA, 10^5 c/μmole (min)	C^{14} conc 10^5 c/ml (min)	SA, 10^5 c/μmole (min)	C^{14} conc 10^5 c/g (min)	SA, 10^5 c/μmole (min)
1	I	4.4	2.2	4.4	2.2	0	0
	F	2.57	2.16	4.37	1.96	4.2	1.81
2	I	0	0	4.4	2.2	0	0
	F	.02	.021	3.0	1.23	2.09	.92
3	I	4.4	2.2	0	0	0	0
	F	2.68	1.42	1.68	.65	2.29	.80

SA = specific activity. I = initial concentration. F = final concentration.
* Both mucosal and serosal fluids contain non-labeled L-tryptophan 2 mM.
† The tissue was extracted with 5 ml distilled water for 24 hr and amount of C^{14} and L-tryptophan was determined.

port study has been tried. The result was inconclusive. Data show here that the same intestinal sac which did not transport glucose actively, transported certain tyrosine analogues and L-tryptophan against a concentration gradient. It is hard to believe that a physical barrier would exist for glucose only, but not for the amino acids. Blood sugar concentration determined on 3 flounders ranged from 26-50 mg%. This may suggest that the controlling system for carbohydrate metabolism in the fish is different from that of mammals and no active transport system for glucose absorption is required.

(b) Lin and Wilson(8) demonstrated the specificity of aromatic amino acid transport mechanism in intestine by showing that the golden hamster intestine transports L-tyrosine, meta-tyrosine, but not D-tyrosine and ortho-tyrosine. This specificity of intestinal transport mechanism was not observed in the flounder. As shown in Table I, L-tyrosine, as well as D-tyrosine, and both the meta and ortho isomers‖ were transported by the flounder intestine from mucosal to serosal side against a concentration gradient. However, flounder intestine does show some selectivity to transport 3,4-dihydroxyphenylalanine (DOPA) and 3,5-dichloro-L-tyrosine, but not the other 3,5-disubstituted analogues. Similar results have been reported in our laboratory(2).

It is well known that in mammals, among the aromatic amino acids, L-tryptophan and L-phenylalanine are essential in the diet; L-tyrosine is not. Logically, a special transport mechanism in the intestine for the essential amino acids is required in our body. Since the indole ring of tryptophan is planar as is the benzene ring of phenylalanine and tyrosine, a gross structural similarity between these amino acids is suggested and a common transport mechanism for these 3 L-amino acids by the intestine has been postulated(9). The literature reveals very little on the composition and biochemical activity of the different amino acids in flounder. Our result shows that L-phenylalanine inhibits the transport of L-tyrosine in flounder intestine, suggesting there may be a common pathway for transport of both L-phenylalanine and L-tyrosine. A specificity of this transport system is certainly not well developed as observed in the mammalian intestine, because the flounder intestine transports D-tyrosine, and ortho-tyrosine as well as the L-tyrosine and meta isomer.

However, the flounder intestine shows a specificity for transport of tryptophan derivatives similar to that in mammals(9), namely only L-tryptophan was transported, and the D-form and decarboxylated compound of tryptophan were not transported.

It has been reported that the sodium ion plays an important role in intestinal transport of tyrosine and tryptophan derivatives (9,10). Our present data show that sodium depletion in the fish Ringer results in an inhibition of tyrosine transport, but this inhibition is not as marked as in mammalian intestine.

Summary. Everted intestinal sacs of winter flounders were used in the summer to study the transmucosal movement of glucose and certain aromatic amino acids and derivatives. It was found that glucose was not transported against a concentration gradient. L-tyrosine, D-tyrosine, *m*-tyrosine, *o*-tyrosine, 3-amino-tyrosine, 3,5-dichlorotyrosine and 3,4-dihydroxyphenylalanine were transported from mucosal to serosal side against a concentration gradient. 3,5-dibromo- and 3,5-dinitro-tyrosine were not transported. Among the tryptophan compounds which were studied, only L-tryptophan was transported; D-tryptophan and tryptamine were not transported against a concentration gradient.

‖ The identity of the tyrosine analogues used in this investigation has been examined with the everted intestinal sacs from 3 hamsters. Results confirmed the finding of Lin and Wilson(8) that hamster intestine transported L-tyrosine and meta-tyrosine, but not the D-tyrosine and ortho isomer.

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Received November 6, 1964. P.S.E.B.M., 1965, v118.

Effect of Chlorothiazide on Rat Liver Glucokinase, Hexokinase and Dihydroxyacetone-Kinase Activities.* (30011)

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Salas, Vinuela and Sols(1) and Sharma, Manjeshwar and Weinhouse(2) reported on a glucose phosphorylating enzyme, glucokinase, the synthesis of which, in contrast to hexokinase, is insulin dependent. This enzyme is virtually absent in the liver of alloxan diabetic rats, but with repeated insulin injections its normal level can be restored in 50 to 100 hours. The activity of another enzyme, dihydroxyacetone-kinase, which phosphorylates dihydroxyacetone (DHA), also has been shown to be insulin dependent(3,4). This enzyme, too, is absent from the liver of the alloxan diabetic rat. With injection of insulin to the diabetic animal, normal enzyme levels can be restored in 20 to 30 hours. A decrease in plasma insulin-like activity has been reported after administration of benzothiadiazine drugs(5). The following studies were carried out to determine if chlorothiazide administration to rats alters liver glucokinase, hexokinase and DHA-kinase activities.

Methods. Eighteen non-fasting male Sprague-Dawley rats weighing 250 to 350 g were given 100 mg chlorothiazide† per kg body weight per day in drinking water for 14 to 18 days. Eighteen similar control animals drank tap water. Animals were sacrificed by decapitation. Glucokinase and hexokinase activities were determined by the method of Salas, Vinuela and Sols(1). Livers were perfused *in situ* with 30 ml of a solution containing 150 mM KCl, 5 mM sodium EDTA, and 5 mM $MgCl_2$ at pH 7.0. They were then removed, weighed and homogenized in a glass-Teflon homogenizer with 2 volumes of cold buffer containing 100 mM Tris-HCl, 1 mM sodium EDTA and 1 mM β-mercaptoethanol at pH 7.4. Homogenates were then centrifuged at 2°C for 30 minutes at 108,000 *g*. Supernatant solutions were assayed for glucokinase and hexokinase activities at room temperature (22-23°C). These assay systems, designated C, contained in 3 ml final volume 0.2 i.u. glucose 6-phosphate dehydrogenase, 0.25 mM NADP, 5 mM ATP, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 50 mM Tris-HCl at pH 7.4, 0.1 M glucose and 0.05 ml freshly prepared supernatant solution. Four additional assay systems were carried out which differed from C as follows: B had 0.5 mM instead of 0.1 M glucose, A had 50 mM N-acetyl-glucosamine instead of 0.1 M glucose, D had 0.25 mg chlorothiazide added per ml, and E had 1 unit of glucagon-free insulin‡ added. C-B gives glucokinase activity and B-A gives hexokinase activity(2). Rates of NADPH formation were divided by 1.7 to correct for the contribution of endogenous 6-phosphogluconate dehydrogenase(2).

For DHA-kinase activity determinations, 22 similar rats were given 100 mg chlorothiazide per kg body weight per day in drinking water for 14 to 18 days and 25 control animals drank tap water. Rats were sacrificed by decapitation and their livers re-

* Supported in part by grants from Michigan Heart Assn. and U.S.P.H.S. #HE-08083-01 from Nat. Inst. Health.

† Kindly supplied as Lyovac Diuril by Merck, Sharp & Dohme Co.

‡ Kindly supplied by Eli Lilly & Co.