Sarkar, P. K., and Doty, P. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 981.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schmid, K. (1953), *J. Am. Chem. Soc. 75*, 60.
Seal, U. S., and Doe, R. P. (1962), *J. Biol. Chem. 237*, 3136.
Seal, U. S., and Doe, R. P. (1964), *Excerpta Med. Ser. No.* 83, 325.
Seal, U. S., and Doe, R. P. (1966), *in* Steroid Dynamics, Pincus, G., Nakao, T., and Tait, J. F., Ed., New York, N. Y., Academic, p 63.
Slaunwhite, W. R., Jr., Lockie, G. N., Back, N., and Sandberg, A. A. (1962), *Science 135*, 1062.
Slaunwhite, W. R., Jr., Schneider, S., Wissler, F. C., and Sandberg, A. A. (1966), *Biochemistry 5*, 3527.
Walborg, E. F., Jr., and Ward, D. N. (1963), *Texas Rept. Biol. Med. 21*, 601.
Warren, L. (1957), *J. Biol. Chem. 234*, 1971.
Weimer, H. E., Mehl, J. W., and Winzler, R. J. (1950), *J. Biol. Chem. 185*, 561.
Westphal, U. (1961), *in* Mechanism of Action of Steroid Hormones, Villee, C. A., and Engel, L. L., Ed., New York, N. Y., Pergamon, p 33.
Westphal, U. (1964), *J. Am. Oil Chemists' Soc. 41*, 481.
Westphal, U. (1966), *Z. Physiol. Chem. 346*, 243.
Westphal, U. (1967a), *Arch. Biochem. Biophys. 118*, 556.
Westphal, U. (1967b), *Methods Enzymol.* (in press).
Westphal, U., and Ashley, B. D. (1962), *J. Biol. Chem. 237*, 2763.
Westphal, U., Ashley, B. D., and Selden, G. L. (1961), *Arch. Biochem. Biophys. 92*, 441.
Westphal, U., and Forbes, R. T. (1963), *Endocrinology 73*, 504.
Westphal, U., Ganguly, M., and Kerkay, J. (1966), *Federation Proc. 25*, 799.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 290.
Winzler, R. J. (1960), *in* The Plasma Proteins, Vol. 1, Putnam, F. W., Ed., New York, N. Y., Academic, pp 309, 318.

The Study of Membrane Function by Observation of the Change in Rate of Transcellular Migration of Amino Acids*

John E. Harris and Leo Friedman

ABSTRACT: The functioning of a membrane may be observed by monitoring the transport of amino acids. We have devised an experimental system in which the factors affecting membrane function and the effects of specific compounds can be studied. A complex membrane of Ehrlich ascites cells on a Millipore filter similar to that described by D. L. Oxender and H. N. Christensen (*J. Biol. Chem. 234*, 2321 (1959)) separates two compartments of an all-glass apparatus. In each compartment is a K^+-free Krebs–Ringer bicarbonate solution containing the amino acid being studied. The larger (45 ml) compartment contains [^{14}C]amino acid, and the rate of accumulation of the radioactive label in the smaller (6.5 ml) compartment is determined by periodic sampling. Under these conditions, the addition of KCl stimulated the rate of migration of radioactive glycine, α-aminoisobutyric acid (AIB), and glutamic acid. The apparent increase in the rate of transcellular migration was the result of an increased rate in the direction of the K^+ rather than a decreased rate in the opposite direction. However, in the case of AIB, there was a decreased rate toward the K^+-containing compartment and also an increased rate in the opposite direction. The presence of 5 mM CN^- (in the glutamic acid system) inhibited the increase in rate from occurring.

Several years ago Oxender and Christensen (1959) reported transcellular concentration was a consequence of intracellular accumulation across a barrier of Ehrlich ascites cells. Their system consisted of a stainless-steel Millipore filter apparatus in which the complex cell membrane was prepared and tested. The bottom of this apparatus was coupled to a bubble-driven circulation device (Ussing and Zerahn, 1951) which formed a 35-ml compartment, and the funnel of the filter formed a 0.5-ml compartment. Oxender placed equal amounts of radioactivity in both compartments and established transcellular concentration when a difference in radioactivity was observed between the two compartments.

The capacity to follow the migration of amino acids

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in a system in which phases on both sides of the membrane are readily accessible was of interest as this offered the possibility of a model in which membrane function could be studied under a variety of controlled situations. Such a system allows observation of the migration of substances as they cross a barrier of biological material and study of the various factors controlling this activity.

Our attempts to use this system as originally described met with several difficulties. The maintenance of the small (0.5 ml) phase over the period of time of the experiment was very delicately tied to the hydrostatic pressure created by the level of aqueous phase in the circulation device. Because of the small size and the design of the system, it was not possible to bubble an oxygen–carbon dioxide gas mixture in the 0.5-ml compartment. Also this limited the size of the aliquot taken for sampling and made accurate sampling very difficult.

We designed an all-glass system which provided circulation in both compartments. A layer of cells is prepared on a 47-mm (5.0-μ pore size) Millipore filter by controlled suction, removed from the filter apparatus, covered with a second Millipore filter forming a sandwich, and placed in the all-glass system separating two compartments, one of 6.5 ml, the other of 45 ml (see Figure 1). This system minimizes the hydrostatic effect, provides circulation in both compartments, and allows accurate samples to be taken. We add radioactivity to compartment B and observe the flow rate by sampling compartment A at selected time intervals. After the addition of a new component (*e.g.*, K^+ to either compartment A or B), we can determine the effect of this addition on rate of migration either toward the treated compartment or away from the treated compartment, respectively.

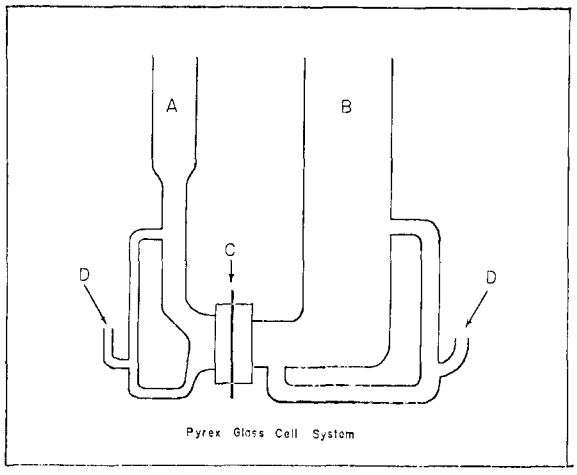

FIGURE 1: Pyrex glass system. The complex Ehrlich ascites cell membrane is placed at C between compartments A (6.5 ml) and B (45 ml) and the 95% O_2–5% CO_2 gas mixture bubbles in at D in each compartment.

Methods

Preparation of an Ehrlich Ascites Cell Membrane. Ehrlich ascites carcinoma cells are maintained by weekly transplantation into the peritoneal cavity of 10-week-old Swiss albino mice, strain Ha/ICR.

Donor mice were sacrificed by cervical dislocation 6–10 days after transplantation of Ehrlich ascites cells. The ascites was poured from the opened abdomens into a sterile dish and then transferred to 20 ml of room temperature potassium-free Krebs–Ringer bicarbonate solution containing an amino acid at 10 mM. The presence of an amino acid at this stage is essential. Hemorrhagic ascites fluids were not used. The cells were centrifuged at 700 rpm for 5 min and the supernatant was decanted. The cells were resuspended in potassium-free Krebs–Ringer bicarbonate solution containing an amino acid. The packed cell volume was measured and adjusted to 3%; 20 ml of this suspension was poured on a 47-mm (5.0-μ pore size) Millipore filter in a Millipore filter holder.[1] After 6 min of suction at approximately 12–18 mm, the residual portion of the suspension was decanted; the cell-coated filter was washed twice by decantation with the K^+-free amino acid buffer solution, removed from the holder, placed in the all-glass apparatus, and covered with a second filter. The complex cell membrane sandwiched between two filters was now ready for the experiment.

Pyrex Glass System. A schematic drawing of the all-glass apparatus is shown in Figure 1. At C, the membrane is held in place by two Millipore Pyrex funnels (XX10-025-04) clamped together with a no. 28 ball-and-socket-joint pinch clamp. The funnels were altered so that upper chamber A consists of 5 cm of 15-mm tubing welded to 5 cm of 8-mm tubing with the bubble-driven circulation device D made of 3-mm tubing. Chamber B is essentially all 25-mm tubing with its circulation device made of 8-mm tubing. Gas (95% O_2–5% CO_2) provided the circulation. The apparatus is 13 cm high.

The experiments were performed with Krebs–Ringer bicarbonate solution containing a labeled or unlabeled amino acid at 10 mM. Isotonicity was maintained at all times. When potassium-free Krebs–Ringer bicarbonate solutions containing an amino acid at 10 mM were used, the sodium chloride content of this solution was adjusted so that the relative concentration of each component remained constant.

Observation of Flow in Both Directions. Observation of the rate of flow in each direction, toward the treated compartment and away from the treated compartment, was accomplished by performing two identical experiments, each with a control. In the first experiment with a labeled amino acid in compartment B and the treatment given to compartment A, we observed the

[1] We are now washing all Millipore filters with 4–20-ml portions of potassium-free Krebs–Ringer bicarbonate solution just before use to minimize the effect of the Triton X-100 which, according to Cahn (1967), is 2–3% by weight of the Millipore. This wash eliminates the slight foaming we had observed.

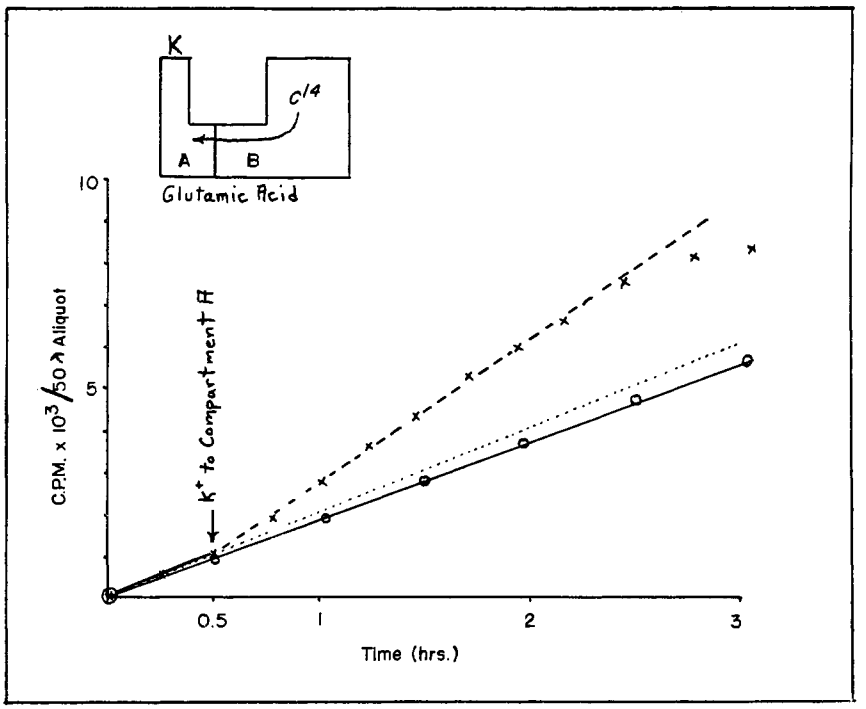

FIGURE 2: Typical glutamic acid rate. Accumulation after the addition of KCl to compartment A at 0.5 hr.

migration of ^{14}C toward the treated compartment. In the second experiment, with the label again in B and the treatment given to B, we observed the migration of ^{14}C away from the treated compartment.

Male Swiss albino mice (strain Ha/ICR, 8-weeks old) were obtained from the Charles River Breeding Laboratories, Wilmington, Mass.

L-[U-^{14}C]Glutamic acid, [1-^{14}C]glycine, and α-[1-^{14}C]-aminoisobutyric acid were obtained from the New England Nuclear Corp., Boston, Mass. L-Glutamic acid, glycine, α-aminoisobutyric acid, pyridoxal hydrochloride, and pyridoxal 5'-phosphate were obtained from Sigma Chemical Co., St. Louis, Mo.

For liquid scintillation counting, a measured aliquot of bathing solution (approximately 0.050 ml) was added to 10 ml of dioxane containing 100 g/l. of naphthalene, 7 g/l. of 2,5-diphenyloxazole, and 0.3 g/l. of 1,4-bis(5-phenyloxazolyl-2)benzene (Snyder, 1964). The resulting clear solutions were counted in an ANSitron liquid scintillation spectrometer Model 1300 using the external standard to determine counting efficiency. The measured counts were 4–4000-fold greater than the background counts, and counting efficiencies were greater than 97%.

The measured aliquots of bathing solution were obtained using one Hamilton Microliter Syringe no. 710-N, 0.1-ml capacity (Burrell Corp., Pittsburgh, Pa.). After about 0.05 ml of solution had been removed, this aliquot was drawn up into the glass barrel and measured by placing one meniscus at 0.01 and reading the volume of solution.

Two systems were used, an experimental and a control, and carefully measured aliquots of approximately 50 μl were taken at 15-min intervals. The counts per minute per sample were adjusted to a standard volume of 50 μl.

The Millipore filters (SM, 5.0-μ pore size, 47-mm diameter) and Millipore filtering apparatus were obtained from Millipore Filter Corp., Bedford, Mass.

Results

When potassium-free Krebs–Ringer bicarbonate medium containing 10 mM amino acid was present on each side of the membrane and the migration of radioactive label into compartment A was followed by periodic sampling, the rate of ^{14}C accumulation was constant. An untreated control such as this was run with almost every experiment. These controls clearly demonstrated that there was no change in slope in the absence of a treatment. It was demonstrated experimentally that the escape curve is linear for at least 5 hr. This is to be expected since the amount of amino acid moving into A is a small fraction of that present in B.

When an experiment was run, both the control and the test membrane were prepared from the same batch of cells with the same solutions. The migration of radioactive amino acid was followed in each system for 30 min. Then the test system was altered so that compartment A was 15 mM in potassium by the addition of isotonic solution of potassium chloride containing 10 mM glutamate. The results of an experiment with glutamic acid shown in Figure 2 are typical. The

TABLE I: Effect of Various Treatments on the Flux of Amino Acids through the Complex Ehrlich Ascites Cell Membrane.

Treatment	Direction of Flow	$Rate_p$: $Rate_a$[a]	n[b]	P Value[c]	(1) Lag in Effect (min) and (2) Duration of Effect (min)
		Glutamic Acid			
K^+	$\rightarrow K^+$	1.64 ± 0.21[d]	7	<0.001	(1) 0, 15, 0, 0, 0, 0, 15 (2) 60, 60, 150, 90, 105, 150, 135
K^+	$\leftarrow K^+$	1.00	2	>0.5	NA[e]
$K^+ + CN^-$	$\rightarrow K^+ + CN^-$	0.62 ± 0.007	2	<0.01 >0.005	(1) 0, 0 (2) 75, 30[f]
$K^+ + CN^-$	$\leftarrow K^+ + CN^-$	0.84 ± 0.07	4	<0.02 >0.01	(1) 0, 15, 0, 15 (2) 150, 165, 150, 120
		Glycine			
K^+	$\rightarrow K^+$	1.32 ± 0.19	6	<0.01 >0.005	(1) 0, 0, 60, 60, 45, 60 (2) 75, 150, 45, 30, 60, 75
K^+	$\leftarrow K^+$	1.00	2	>0.5	NA
Pyridoxal 5′-phosphate	→PP	1.21 ± 0.08	3	<0.05 >0.025	(1) 0, 0, 0 (2) 90, 75, 90
Pyridoxal 5′-phosphate	←PP	1.00	3	>0.5	NA
Pyridoxal hydrochloride	→P-HCl	1.00	3	>0.5	NA
Pyridoxal hydrochloride	←P-HCl	1.00	2	>0.5	NA
		Aminoisobutyric Acid (AIB)			
K	→K	0.72 ± 0.007	2	<0.02 >0.01	(1) 90, 60 (2) 60, 60
K	←K	1.29 ± 0.15	4	<0.05 >0.025	(1) 0, 0, 0, 60 (2) 90, 90, 150, 75
Controls (no treatment)		1.00	29	NA	NA

[a] Rate after treatment:rate before treatment. [b] Number of experiments performed. [c] Probability that the observed effect is due to chance. [d] Plus and minus standard deviation. [e] Not applicable. [f] CN^- solution was several days old.

first three points of the experimental membrane are used to determine the projected slope or projected rate of migration of radioactivity over the 3-hr experiment. The control run with each experiment supports the conclusion that the response to a particular treatment was due to that treatment and not to the behavior of the cells. In Figure 2 there is no lag before the increase in slope, and the duration of the effect (*i.e.*, change in slope) is 120 min. Comparisons of the flux after treatment with the initial rate as shown by the calculated ratios of slope after treatment to initial slope are tabulated in Table I. With glutamic acid an increase in rate of migration through the membrane was observed toward the treated compartment.

In experiments where the potassium was added to compartment B, the rate of migration of radioactivity remained unchanged in both the treated and control systems. The increased rate in one direction indicated that glutamate must migrate against a concentration gradient. The results using radioactive glycine were similar to those with glutamate.

The experiments with AIB[2] gave different results. When compartment A was treated with potassium, the flow of radioactivity toward the treated compartment remained constant for at least 60 min and then gradually decreased until the slope was an average of 0.72 ± 0.007 times the original slope. When the treatment is in compartment B, the rate of radioactivity away from the K^+ increases until it is 1.29 ± 0.15 times greater than the projected rate. The result of the increased rate in one direction and decreased rate in the other indicates the accumulation of AIB (Figure 3) away from the K^+. This effect is opposite to that seen with glutamic acid and glycine.

In experiments with pyridoxal 5′-phosphate, normal Krebs–Ringer bicarbonate solution containing 10 mM glycine was used. The average increased rate was 1.21 ± 0.08 times the projected value toward the pyridoxal 5′-phosphate containing compartment. No change in rate away from the treated compartment indicated an uphill flow of glycine toward the treated compartment. Similar studies with pyridoxal hydrochloride produced no change in flow of [^{14}C]glycine.

The variability of membranes made from the same batch of cells at approximately the same time was

[2] Abbreviation used: AIB, α-aminoisobutyric acid.

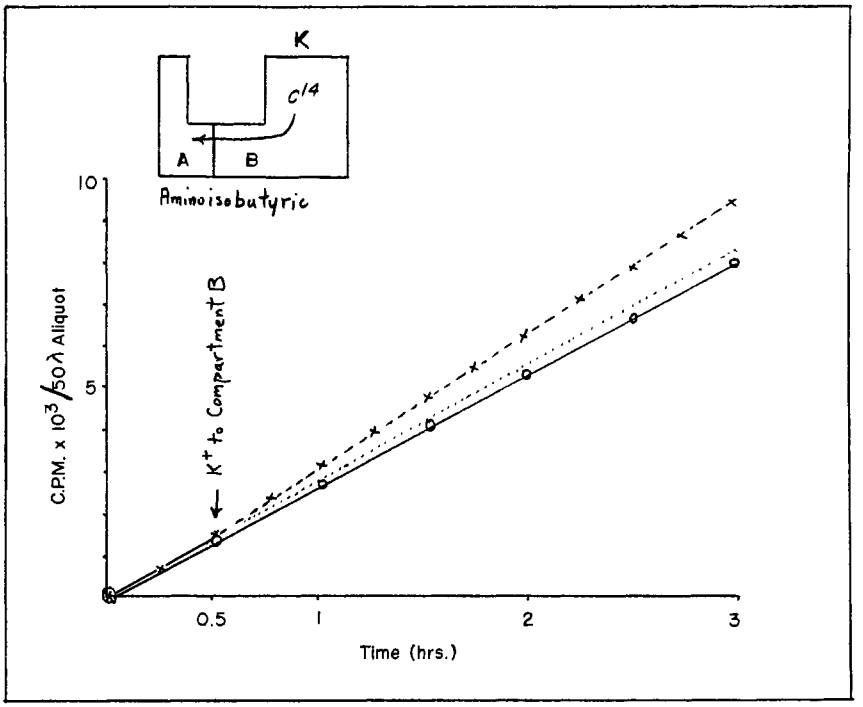

FIGURE 3: Typical aminoisobutyric acid rate. Accumulation after the addition of KCl to compartment B at 0.5 hr.

studied by determining the ratio of the initial slope of the experimental membrane to the slope of the corresponding control membrane. These ratios, ranging from 0.43 to 2.03 with an average value of 1.06 ± 0.33 and a calculated P value between 0.3 and 0.4, indicated that usually there was no significant difference between two membranes prepared at the same time.

The effect of cyanide on the potassium system is shown in the following experiments in which each of the two systems was altered at 0.5 hr, as indicated. In the first system compartment A was made 15 mM in potassium and 5 mM in cyanide, and the second system received only the potassium and served as a control. The cyanide-containing system shows a marked decrease in rate of [^{14}C]glutamate accumulation as shown in Table I, whereas the control system reacted normally to the addition of K^+ with an increase in rate of flow toward the K^+ as compared to the rate before K^+ treatment. When the cyanide was added to compartment B, the rate of flow away from the $K^+ + CN^-$ was also decreased although to a somewhat lesser extent.

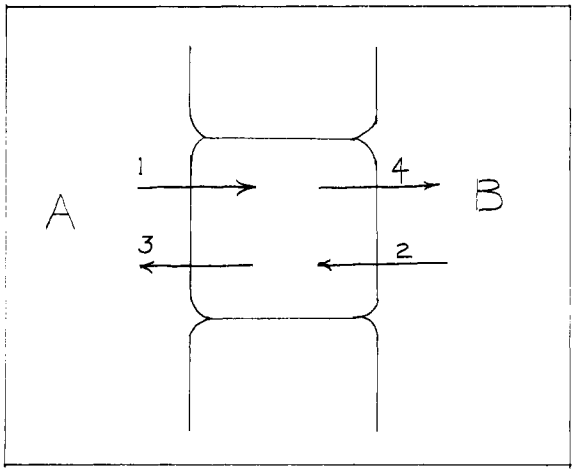

FIGURE 4: Diagram representing the influx (1 and 2) and efflux (3 and 4) rates of an amino acid during membrane experiments between compartments A and B.

Discussion

Cyanide inhibits transport fluxes but does not inhibit exchange fluxes of amino acids by depression of cellular energy metabolism in Ehrlich ascites cells (Jacquez and Sherman, 1965). The results indicate that not only is the K^+ effect dependent on cellular metabolism but that a component of the initial transmigration rate is also.

Potassium alters the relationship of the influx and efflux rates 1–4 (see Figure 4).

Before the addition of a new component, the rates are 1 + 4 = 2 + 3. However, the addition of K^+ results in a change in one or more of these rates and the accumulation of the amino acid in the K^+-containing compartment in the case of glycine and glutamic acid and in the opposite compartment in the case of AIB.

The use of ^{14}C measurements to follow the migration of amino acids assumes that no alteration occurs during passage through the cells. Ehrlich cells concentrate many amino acids rapidly and extensively, and only a few, *e.g.*, leucine, glutamine, and arginine are rapidly metabolized (Quastel, 1965). AIB is an unnatural amino acid and is not metabolized (Noall *et al.*, 1957).

Christensen *et al.* (1952b,c) associated the accumulation of amino acids with the loss of cell potassium. For optimal transport of amino acids into Ehrlich ascites cells, K^+ is required in the extracellular fluid (Christensen *et al.*, 1952a). On the other hand, Hempling and Hare (1961) suggest that amino acids (such as glycine) facilitate potassium transport rather than the reverse. Possibly different amino acids affect potassium transport in different ways. At present, not enough information is at hand to speculate on what happens to the individual rates 1–4. The system here described offers the possibility of studying these different rates by including measurements of the intracellular compartment C. The clarification of the individual rates will lead to a clearer picture of the function of the Ehrlich ascites cell membrane in the transport and exchange diffusion of amino acids under various conditions.

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References

Cahn, R. D. (1967), *Science 155*, 195.
Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M. (1952a), *J. Biol. Chem. 198*, 1.
Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M. (1952b), *J. Biol. Chem. 198*, 17.
Christensen, H. N., Riggs, T. R., Rafu, M. L., Ray, N. E., and Palatine, I. M. (1952c), *J. Biol. Chem. 197*, 57.
Hempling, H. G., and Hare, D. (1961), *J. Biol. Chem. 236*, 2498.
Jacquez, J. A., and Sherman, J. H. (1965), *Biochim. Biophys. Acta 109*, 128.
Noall, M. W., Riggs, T. R., Walker, L. M., and Christensen, H. N. (1957), *Science 126*, 1002.
Oxender, D. L., and Christensen, H. N. (1959), *J. Biol. Chem. 234*, 2321.
Quastel, J. H. (1965), *Proc. Roy. Soc. (London) B163*, 169.
Snyder, F. (1964), *Anal. Biochem. 31*, 183.
Ussing, H., and Zerahn, K. (1951), *Acta Physiol. Scand. 23*, 110.

Comparison of Guinea Pig γ_1- and γ_2-Immunoglobulins by Peptide Mapping*

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ABSTRACT: To enable comparison of two classes of immunoglobulins from the same animal species γ_1- and γ_2-globulins were obtained from guinea pig anti-2,4-dinitrophenyl antibodies. The fragments produced by the action of papain and pepsin, and the H and L chains resulting from extensive reduction were examined by peptide mapping after digestion with trypsin. These two classes of immunoglobulins contain similar Fab and $F(ab')_2$ fragments, identical L chains, half-similar H chains, and dissimilar Fc fragments. It is concluded that their Fd fragments are closely related and that class differences are localized largely to the Fc portions of the H chains. The Fc portions do, however, contain a number of similar peptides.

Guinea pigs offer certain advantages for comparing different classes of immunoglobulins within a single animal species. γ_1- and γ_2-globulin antibodies can be obtained in reasonable yields and relative purity (Benacerraf *et al.*, 1963), and for structural studies it is fortunate that both classes can be successfully subjected to standard procedures for preparing fragments by proteolysis and polypeptide chains by reduction and gel filtration. The fact that the Fc fragments of γ_1- and γ_2-globulins mediate distinct biological phenomena (Ovary *et al.*, 1963; Bloch *et al.*, 1963; Berken and Benacerraf, 1966) is of additional interest.

It is known that individual classes of immunoglobulins within a given animal species differ with regard to their Fc fragments but share common light poly-

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