*Proc. Natl. Acad. Sci. USA*
Vol. 80, pp. 6101–6104, October 1983
Medical Sciences

# Ouabain-like activity in human cerebrospinal fluid

(Na$^+$/K$^+$-ATPase/Na$^+$/K$^+$-ATPase inhibitor/Na$^+$ transport inhibitor/natriuretic factor)

JOSÉ HALPERÍN*, ROSITA SCHAEFFER*, LUIS GALVEZ*, AND SALVADOR MALAVÉ†

*Unidad de Investigaciones and †Unidad de Radiodiagnóstico, Centro Médico Docente la Trinidad, A.P. 80474, Caracas 108, Venezuela

*Communicated by Joseph F. Hoffmann, June 27, 1983*

**ABSTRACT** **Human cerebrospinal fluid has been found to mimic the effect of ouabain on net Na$^+$ efflux and $^{86}$Rb$^+$ influx across erythrocyte membranes and on the *in vitro* activity of a purified Na$^+$/K$^+$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) derived from canine kidney. These results indicate the possible existence in human cerebrospinal fluid of an endogenous factor with ouabain-like activity, which might be linked to sodium metabolism.**

Since the classic cross-circulation experiments of de Wardener *et al.* (1) and Dahl *et al.* (2), numerous investigators have provided experimental evidence supporting the view that a humoral factor could influence urinary sodium excretion and vascular responsiveness by inhibition of the Na$^+$/K$^+$ pump (3, 4). Although the chemical nature and site of production of this putative hormone have remained obscure, some data suggest the brain as the possible source (5).

Neurochemical analysis of cerebrospinal fluid (CSF) is recognized as a method that can provide valid information about the metabolism of neurotransmitters and hormones produced in the central nervous system of living subjects (6). We report here the presence of an endogenous "ouabain-like" activity in human cerebrospinal fluid.

## MATERIAL AND METHODS

**CSF Samples.** CSF was obtained by lumbar puncture from healthy female and male donors to whom lumbar myelographies were performed as a diagnostic procedure for possible spinal-root compression. Informed consent was obtained in all cases. CSF samples were clear and colorless and had normal biochemistry and cell count. No erythrocytes were detected.

The Na$^+$ and K$^+$ contents of each CSF sample were determined by flame photometry. The final concentration of both ions was adjusted to a value similar to the control medium of the respective experiment by adding the required amount of a 1 M NaCl or KCl solution.

CSF Ca$^{2+}$ and Mg$^{2+}$ contents were determined on an ACA II analyzer (DuPont), and vanadium was determined by flameless atomic absorption spectrometry (Varian GTA-95 Atomizer coupled to a Varian 875 spectrometer; maximum sensitivity, 25 ppb).

**Assays for Ouabain-like Activity.** *Intracellular Na$^+$ concentration of unloaded human erythrocytes.* Freshly drawn human venous blood, collected in heparin-treated tubes, was centrifuged (1,700 × *g* for 10 min), and the plasma and buffy coat were removed. The erythrocytes then were washed three times with 10 volumes of ice-cold 110 mM $MgCl_2$ and were resuspended in the same solution to obtain a hematocrit of ≈60%. The erythrocyte suspension was distributed in duplicate tubes at a final hematocrit of 5% in a volume of 250 μl of either (*i*) control medium (150 mM NaCl/2.8 mM KCl/1 mM $MgCl_2$/10 mM glucose/5 mM sodium phosphate, pH 7.4 at 37°C) with and without ouabain (0.1 mM) or (*ii*) CSF (with adjusted Na$^+$, K$^+$, and buffer concentrations similar to those of the control medium). An aliquot of the cell suspension was set aside for measurement of the basal intracellular Na$^+$, hemoglobin, and hematocrit.

Samples were incubated for 1, 2, and 3 hr at 37°C and then were washed three times with 10 volumes of isotonic solution of choline chloride (150 mM choline chloride/10 mM Tris·HCl, pH 7.4 at 37°C) with ouabain (0.02 mM). After the last wash, the pellet was hemolyzed with 2.5 ml of Acationex (0.02%) for hemoglobin determination and Na$^+$ measurement in the same sample.

Na$^+$ was measured by atomic absorption spectrometry (Techtron AA4; Melbourne, Australia) and hemoglobin was spectrometrically determined as oxyhemoglobin (541 nm). Intracellular Na$^+$ concentrations were calculated in mmol/liter of erythrocytes and expressed as the ratio of postincubation to preincubation values (relative variation).

*Intracellular Na$^+$ concentration of preloaded erythrocytes.* Washed erythrocytes were loaded with Na$^+$ by incubation at 4°C for 20 hr in a loading solution (60 mM NaCl/200 mM choline chloride/3 mM KCl/1 mM $MgCl_2$/2.5 mM sodium phosphate/1 mM EGTA/0.02 mM *p*-chloromercuribenzene sulphonate, pH 7.2 at 4°C) and then were incubated at 37°C for 1 hr in a recovery solution (150 mM NaCl/1 mM $MgCl_2$/5.4 mM sodium phosphate/4 mM cysteine/2 mM adenine/3 mM inosine/1 mM EGTA/10 mM glucose, pH 7.2 at 37°C) as described by Dagher and Garay (7). After recovery, the erythrocytes were washed three times with a cold solution of 110 mM $MgCl_2$, resuspended in the same solution to an hematocrit of ≈60%, and distributed in control, ouabain-containing, or CSF media for further processing as described above.

An aliquot of the loaded-cell suspension together with an aliquot of cells untreated with *p*-chloromercuribenzene sulfonate were set aside to measure intracellular Na$^+$, hemoglobin, and hematocrit. Intracellular Na$^+$ concentrations were calculated in mmol/liter of erythrocytes and were expressed as the relative variation with respect to the initial (preload) cellular Na$^+$ content.

*Rubidium-86 uptake into human erythrocytes.* Rb$^+$ is an ion handled similarly to K$^+$, and therefore $^{86}$Rb$^+$ uptake by erythrocytes has been used to give a measure of the K$^+$ influx into erythrocytes. Washed erythrocytes were resuspended at a final hematocrit of 5% in a volume of 200 μl of either (*i*) control medium (150 mM NaCl/5 mM KCl/10 mM Tris·HCl, pH 7.4 at 37°C) with and without ouabain (0.1 mM) or (*ii*) CSF (with adjusted Na$^+$, K$^+$, and buffer concentrations similar to those of the control medium). Triplicate aliquots were incubated at 37°C

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: CSF, cerebrospinal fluid.

for 1 and 2 hr with $\approx 10^5$ cpm of $^{86}Rb^+$ (New England Nuclear; specific activity, 1.09 mCi/mg; 1 Ci = 37 GBq) and then washed three times with 10 volumes of ice-cold isotonic choline chloride. Radioactivity was eluted with 300 μl of 5% trichloroacetic acid and assayed in 5 ml of isotonic saline in a Searle liquid scintillation spectrometer (8). Results were calculated in cpm per $10^9$ cells.

*Assay for $Na^+/K^+$-ATPase inhibition.* The $Na^+/K^+$-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activity was estimated by the rate of [$\gamma$-$^{32}$P]ATP hydrolysis. Highly purified canine kidney $Na^+/K^+$-ATPase (grade IV, lot 22F-9525), free of $Mg^{2+}$-ATPase activity, was purchased from Sigma. It was dissolved in 20 mM Tris·HCl, pH 7.4/1 mM EDTA at a protein concentration of 6.1 mg/ml. For each assay the enzyme was further diluted 1:10 in 20 mM Tris·HCl, pH 7.4/5.2 mM $MgCl_2$/0.2 mM EDTA. The enzyme (50 μl) was assayed in a final volume of 1 ml of either (*i*) control medium (140 mM NaCl/20 mM KCl/5.2 mM $MgCl_2$/1 mM EDTA/50 mM Tris·HCl, pH 7.4 at 37°C) with and without 0.1 mM ouabain or (*ii*) CSF (with adjusted $Na^+$, $K^+$, and buffer concentrations similar to those of the control medium). After 20 min of preincubation at 37°C, the reaction was started by adding [$\gamma$-$^{32}$P]ATP [enzymatically prepared as described by Walshet and Johnson (9)] dissolved in 20 mM Tris·HCl with 40 mM ATP (vanadium free, Sigma) to a final concentration of 2 mM ATP and ≈250,000 cpm in the reaction mixture. Aliquots of 0.1 ml were separated at 5, 10, 15, and 20 min and immediately diluted in 0.9 ml of ice-cold 20 mM Tris·HCl (pH 8) containing activated charcoal (2 mg/ml); samples were then centrifuged for 10 min at 1,700 × *g*, and 0.5 ml of the supernatant fluid was carefully removed for assay of radioactivity in a Searle scintillation spectrometer (Cerenkov radiation) to determine the liberated $^{32}P_i$. [$\gamma$-$^{32}$P]ATP spontaneous hydrolysis represented <4% of the total counts added. Results were calculated as the percentage of radioactivity (cpm) present in the supernatant fluid with respect to the total radioactivity added and were expressed as the percentage [$\gamma$-$^{32}$P]ATP hydrolysis.

## RESULTS

**Unloaded Erythrocytes.** As expected, the cellular $Na^+$ concentration remained constant when human erythrocytes were incubated in control medium. Incubation of erythrocytes either with CSF or with 0.1 mM ouabain significantly increased their intracellular $Na^+$ concentration with respect to the initial $Na^+$ content (Fig. 1*a*).

**Preloaded Erythrocytes.** When erythrocytes were incubated in a solution containing *p*-chloromercuribenzene sulfonate, their $Na^+$ concentration increased 1.35 times (from 9.8 to 13.5 mmol per liter of erythrocytes; see Fig. 1*b*). These $Na^+$-loaded erythrocytes recovered their preload $Na^+$ content after incubation for 3 hr in control medium. The preloaded erythrocytes incubated in CSF or ouabain-containing media not only failed to recover the initial $Na^+$ concentration but also further increased their intracellular $Na^+$ level.

**$^{86}Rb^+$ Uptake into Human Erythrocytes.** $^{86}Rb^+$ uptake into human erythrocytes was significantly inhibited by incubation of erythrocytes in CSF or in a medium containing 0.1 mM ouabain (Fig. 2). The $Rb^+$ ($K^+$) influx calculated from data shown in Fig. 2 was 2.8 mmol/liter of erythrocytes per hr.

**$Na^+/K^+$-ATPase Inhibition.** The $Na^+/K^+$-ATPase activity is expressed as the rate of [$\gamma$-$^{32}$P]ATP hydrolysis, which remained constant during the experimental time (Fig. 3). The ATPase activity was completely suppressed by 0.1 mM ouabain and was depressed in a dose-dependent manner by incubation in CSF (Fig. 3 *Inset*). CSF diluted 1:8 showed no inhibitory effect. This inhibition was not due to (*i*) calcium ions (because $Ca^{2+}$ concentration in the CSF samples used ranged between 0.9 and 1.1 mM, and the addition of 1 mM EGTA did not result in a sig-

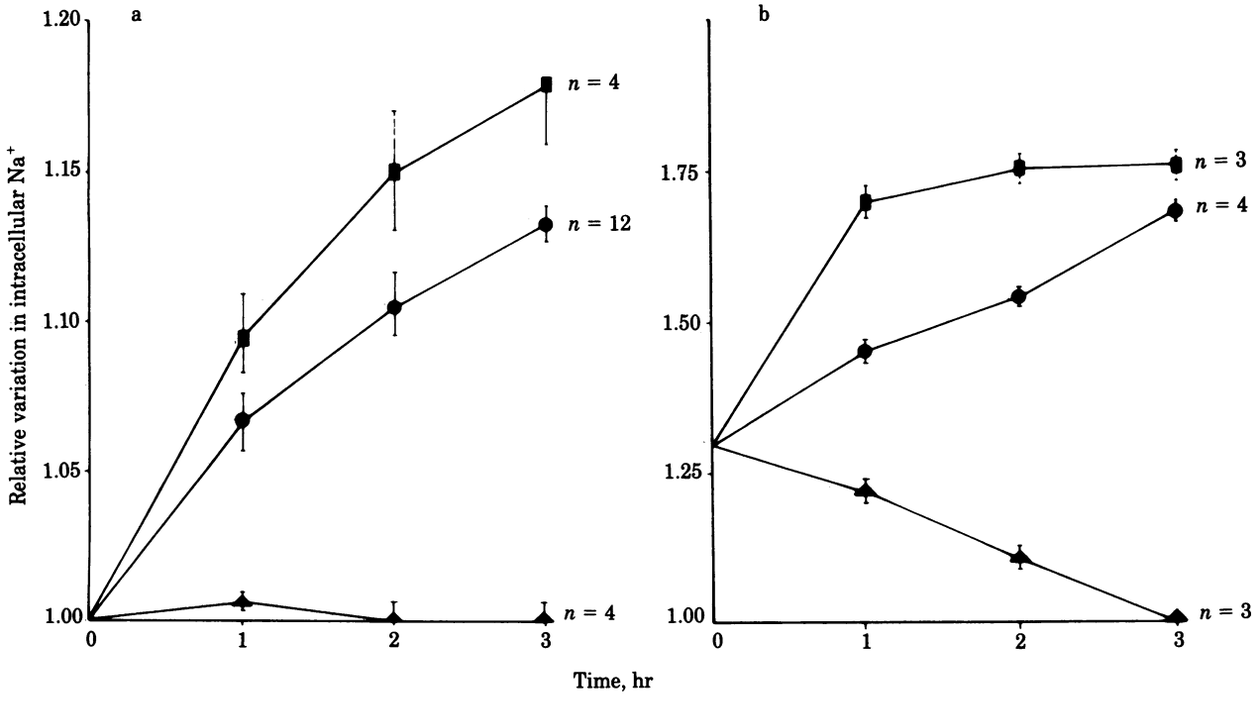


FIG. 1. Increased intracellular $Na^+$ concentration induced by human CSF in erythrocytes. Unloaded (*a*) and preloaded (*b*) human erythrocytes were incubated at 37°C in control (▲), 0.1 mM ouabain-containing (■), and CSF (●) media, and the intracellular $Na^+$ concentration was measured after 1, 2, and 3 hr. The initial $Na^+$ content was 9.8 ± 0.7 mmol per liter of erythrocytes. The zero time point in *b* represents the intracellular $Na^+$ content after loading with *p*-chloromercuribenzene sulfonate. Results are expressed as the relative variation of the intracellular $Na^+$ concentration with respect to the initial $Na^+$ content. Data are corrected for changes in cell volume and reported as the mean ± SEM of the number of experiments, *n*.

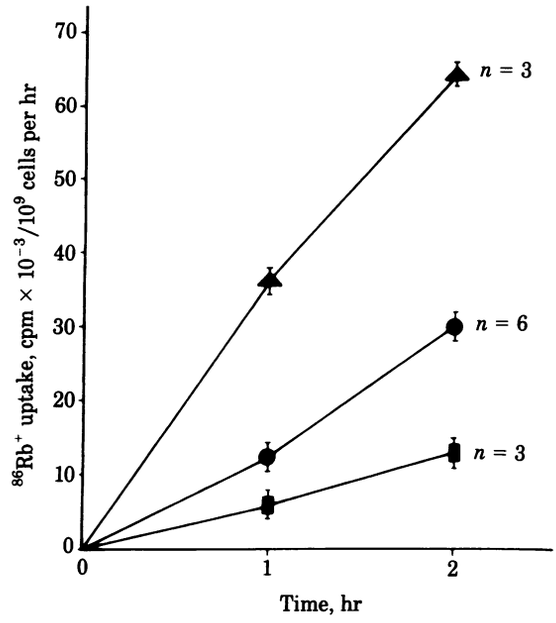


FIG. 2. Inhibition of $^{86}Rb^+$ uptake into erythrocytes by human CSF. Human erythrocytes were incubated at 37°C for 1 and 2 hr in control (▲), 0.1 mM ouabain-containing (■), and CSF (●) media with ≈$10^5$ cpm of $^{86}Rb^+$. $^{86}Rb^+$ uptake into erythrocytes was calculated in cpm per $10^9$ cells. Data are reported as the mean ± SEM of the number of experiments, *n*.

nificant variation of the CSF inhibitory effect) or (*ii*) the presence of vanadium ions (because V was not detected in all of the CSF samples investigated by flameless atomic absorption spectrometry).

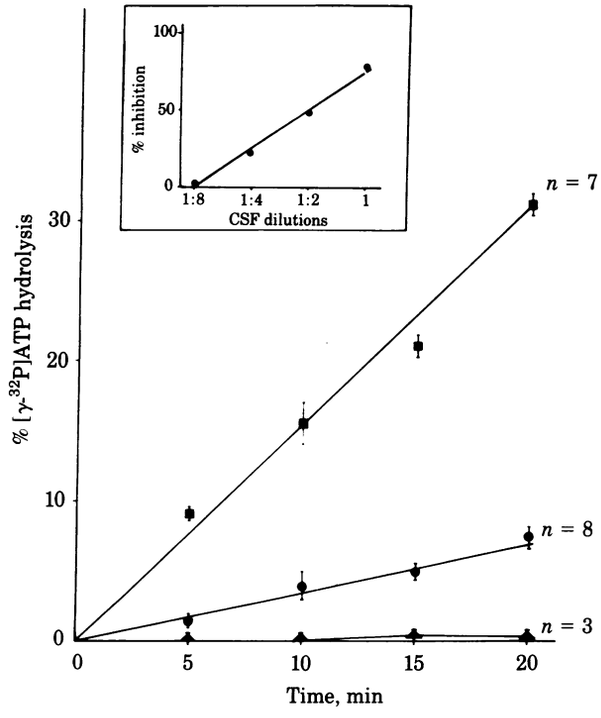


FIG. 3. *In vitro* inhibition of a purified $Na^+/K^+$-ATPase by human CSF. $Na^+/K^+$-ATPase activity was assayed by the rate of [$\gamma$-$^{32}$P]ATP hydrolysis in control (■), ouabain-containing (▲), and CSF (●) media. Results are expressed as the percentage [$\gamma$-$^{32}$P]ATP hydrolysis with respect to the total radioactivity added to the reaction mixture. (*Inset*) Percent inhibition of the $Na^+/K^+$-ATPase activity produced by different CSF concentrations.

Table 1. Effect of human CSF on erythrocyte intracellular $Na^+$ and $^{86}Rb^+$ uptake

| Incubation medium | *n* | Intracellular $Na^+$ increase,* % | $^{86}Rb^+$ Uptake,† % |
|---|---|---|---|
| Control | 4 | 0 | 100 |
| Control/0.1 mM ouabain | 4 | 18 ± 2 | 18 ± 3 |
| CSF (nondiluted) | 16 | 13.8 ± 1.5 | 45.1 ± 4 |
| CSF (diluted 1:2) | 6 | 6.4 ± 1.9 | 70.6 ± 3 |
| CSF (diluted 1:4) | 6 | 2.2 ± 0.7 | 92.5 ± 3 |
| CSF (diluted 1:8) | 6 | 0 | 100 |
| CSF/0.1 mM ouabain | 6 | 17.4 ± 2.8 | 18 ± 2.5 |
| CSF (boiled)‡ | 4 | 14.1 ± 2.3 | 46 ± 3 |
| CSF (trypsin)‡ | 4 | 14.1 ± 2.2 | 45 ± 2.8 |

* Intracellular $Na^+$ concentration after 3 hr was calculated in mmol of erythrocytes per liter, and the results are expressed as the percentage variation with respect to the initial $Na^+$ content (9.8 ± 0.7 mmol/liter of erythrocytes).

† $^{86}Rb^+$ uptake after 2 hr was calculated in cpm per $10^9$ cells, and the results are expressed as the percentage uptake during incubation in control medium, which was taken as 100%.

‡ CSF samples were incubated for 1 hr at 37°C with or without trypsin (1 mg/ml; Sigma). After the incubation period, all reaction mixtures were boiled for 15 min and then assayed for ouabain-like activity.

As shown in Table 1, the CSF also affected the erythrocytes intracellular $Na^+$ concentration and $^{86}Rb^+$ uptake in a dose-dependent manner. The CSF effect on the ion fluxes was not additive with the action of 0.1 mM ouabain and remained unaltered after boiling or trypsin digestion.

## DISCUSSION

The effect of the CSF on the intracellular $Na^+$ concentration in $Na^+$-loaded and -unloaded erythrocytes suggests the possible inhibition by CSF of the uphill extrusion of internal $Na^+$, catalyzed by the $Na^+/K^+$ pump. The $Na^+/K^+$ pump exchanges internal $Na^+$ for external $K^+$; therefore, if this pump is inhibited by CSF, a reduction in the $K^+$ influx also should be expected upon incubation of erythrocytes in CSF. That this seems to be the case is strongly suggested by the ability of CSF to inhibit the $^{86}Rb^+$ uptake into human erythrocytes. As expected, ouabain (0.1 mM) inhibits both $Na^+$ extrusion and $^{86}Rb^+$ uptake.

The above-mentioned CSF effect on erythrocyte ion fluxes was not additive with the action of a maximal inhibitory concentration of ouabain (0.1 mM), indicating that, under our experimental conditions, CSF suppresses the ouabain-sensitive ion fluxes across human erythrocyte membranes.

Both ouabain-sensitive $Na^+$ efflux and $^{86}Rb^+$ uptake provide an index of the $Na^+/K^+$ pump activity (10) and its enzymatic machinery, the $Na^+/K^+$-ATPase that is specifically inhibited by the cardiac glycoside ouabain. That the reduced pump activity shown after incubation of erythrocytes with CSF may be secondary to the suppression of the $Na^+/K^+$-ATPase is strongly supported by the inhibitory effect of human CSF on a highly purified $Na^+/K^+$-ATPase system (Fig. 3).

The results of the experiments reported here clearly suggest the existence in human CSF of an endogenous factor that could be similar to the ouabain-like compound recently extracted from mammalian brain (11–13) or postulated to be present in the plasma of volume-expanded animals (14–16) and some hypertensive subjects (17, 18). Like these compounds, the CSF factor seems to have, at least *in vitro*, ouabain-like activity on $Na^+/K^+$-ATPase from different sources.

The inhibition of the $Na^+/K^+$-ATPase by the digitalic glycosides is thought to be responsible for their pharmacologic properties, such as the positive inotropic action on cardiac mus-

cle and the constrictive effect on blood vessels (19, 20). Some of these pharmacologic properties are similar to the physiologic responses to salt loading, which increases cardiac output, stroke volume, and peripheral resistance (4).

It is tempting to speculate that the ouabain-like activity found in human CSF would represent the action of a compound of still unknown structure and source, which could be the link between high sodium intake and some of the above-mentioned responses to sodium load.

This hypothesis, as well as the biochemical characterization of the responsible molecule, and its possible relationship with the pathological increase of arterial blood pressure seen in sodium-loaded susceptible animals (4) or in subjects with functional disorders of the $Na^+$ and $K^+$ transport systems (21–23) requires further investigation.

The authors are grateful and very much indebted to Dr. R. P. Garay, Dr. G. Whittembury, Dr. M. Piras, and R. Piras for their valuable advice, critical discussions, and review of the manuscript. We also wish to express our sincere gratitude to Dr. Pablo Pulido for his continuous interest and support. We thank Mr. Lorenzo Davalos for [$\gamma$-$^{32}$P]ATP synthesis, Ing. H. Buenafama from INTEVEP S.A. for vanadium detection, and Evelyn Irizarry and Gloria Osorio for expert secretarial and technical assistance. This work was supported by the Centro Médico Docente La Trinidad.

1. de Wardener, H. E., Mills, I. H., Clapham, W. F. & Hayter, C. (1961) *Clin. Sci.* **21,** 249–258.
2. Dahl, L. K., Knudsen, K. D. & Iwai, J. (1969) *Circ. Res.* **25,** Suppl. 1, 21–33.
3. Haddy, F. J. & Overbeck, H. W. (1976) *Life Sci.* **19,** 935–948.
4. Haddy, F. J. (1980) *Am. J. Med.* **69,** 746–758.
5. Bealer, S., Haywood, J. R., Johnson, A. K., Gruber, K. A., Buckalew, V. M. & Brody, M. J. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 1232 (abstr.).
6. Cubeddu, L. & Goldstein, D. J. (1981) in *Perspective in Cardiovascular Research,* eds. Buckley, J. P. & Ferrario, J. M. (Raven, New York), Vol. 6, pp. 153–160.
7. Dagher, G. & Garay, R. P. (1980) *Can. J. Biochem.* **58,** 1069–1074.
8. De Luise, M., Blackburn, G. L. & Glier, J. S. (1980) *N. Engl. J. Med.* **303,** 1017–1022.
9. Walshet, T. F. & Johnson, R. A. (1979) *Biochim. Biophys. Acta* **526,** 11–31.
10. Glynn, I. M. & Karlish, S. J. O. (1975) *Annu. Rev. Physiol.* **37,** 13–55.
11. Haupert, G. T., Jr., & Sancho, J. M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4658–4660.
12. Fishman, M. C. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4661–4663.
13. Lichtstein, D. & Samuelov, S. (1980) *Biochem. Biophys. Res. Commun.* **96,** 1518–1523.
14. de Wardener, H. E. (1977) *Clin. Sci. Mol. Med.* **53,** 1–8.
15. Bricker, N. S., Favre, H., Fine, L. & Bourgoignie, J. J. (1975) *Yale J. Biol. Med.* **48,** 293–303.
16. Gruber, K. A., Witaker, J. M. & Buckalew, V. M. (1980) *Nature (London)* **287,** 743–745.
17. Poston, L., Sewell, R. B., Wilkinson, S. P., Richardson, P. J., Williams, R., Clarkson, E. M., Mac Gregor, G. A. & de Wardener, H. E. (1981) *Br. Med. J.* **282,** 847–849.
18. Haddy, F. J., Pamnani, M. B. & Clough, D. L. (1979) *Life Sci.* **24,** 2105–2117.
19. Leonard, E. (1957) *Am. J. Physiol.* **189,** 185–190.
20. Blaustein, M. P. (1977) *Am. J. Physiol.* **232,** c165–c173.
21. Garay, R. P., Dagher, G., Pernollet, M. G., Devynck, M. A. & Meyer, P. (1980) *Nature (London)* **284,** 281–283.
22. Halperin, J. A. & Galvez, L. (1981) *Acta. Cient. Venez. Supl.* **32,** 196.
23. Canessa, M., Adragna, N., Solomon, H. S., Conolly, T. M. & Tosteson, D. C. (1980) *N. Engl. J. Med.* **302,** 772–776.