Purification from human plasma of endogenous sodium transport inhibitor(s)[1]

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Summary. Na^+,K^+-ATPase inhibitors extracted from plasma of healthy human subjects displaced ^3H-ouabain binding to human erythrocytes and inhibited the Na^+ efflux catalyzed by the Na^+,K^+-pump and unexpectedly the Na^+,K^+-cotransport system without alteration of the Na^+,Na^+-exchange or the Na^+ passive permeability. This suggests the presence in healthy human plasma of endogenous factors with ouabain-like and furosemide-like activities.

Key words. Plasma, human; erythrocytes; Na^+-transport; ouabain; Na^+K^+-pump; NaK-ATPase inhibitors.

An endogenous Na^+,K^+-ATPase inhibitor[3] was shown to be increased in some diseases, such as uremia[4], hepatic failure[5] and hypertension[6,7]. Haddy et al. studying various types of Na^+-dependent experimental hypertension, observed a decrease in ouabain-sensitive sodium flux in blood vessels from hypertensive animals[8] which could be mediated by this endogenous Na^+,K^+-ATPase inhibitor. These authors[8] and Blaustein[9] proposed that such an inhibitor could, by its hemodynamic effects contribute to the rise in blood pressure[9,10]. This hypothesis was extended to human essential hypertension by De Wardener and Mac Gregor[10]. Direct measurement in human plasma of an inhibitor of this kind by its property of inhibiting Na^+,K^+-ATPase activity[6,10,12] and ^3H-ouabain binding[11] was recently reported in essential hypertension. Various attempts to purify and characterize this endogenous factor from various tissues were performed[12-14]. We report here a purification procedure for Na^+,K^+-ATPase inhibitor(s) from human plasma. This purification was made using previously described biochemical properties of the natriuretic factor: a) inhibition of Na^+,K^+-ATPase activity[3,6,11], b) inhibition of ^3H-ouabain binding[11], c) cross reactivity with antidigoxin antibodies[12]. It was found that the plasma extracts could inhibit the Na^+,K^+-pump and also, unexpectedly, the Na^+,K^+-cotransport system in human erythrocytes.

Methods. 10 µl of dog kidney Na^+,K^+-ATPase (2 µg of protein, E.C. 3.6.1.3 Sigma) were incubated at 37°C for 1 h with 100 µl of incubating buffer 80 mM Tris-HCl, 1 mM EGTA, 2 mM ATP (vanadate free, Sigma), 0.02 µCi/ml γ^{32}P-ATP (3000 Ci/mmole, Amersham), 3 mM $MgCl_2$, 100 mM NaCl, 5 m KCl, pH 7.4) in the presence of 10 µl of the plasma fractions to be tested. Blanks were obtained by parallel incubation performed in the presence of 0.1 mM ouabain and in the absence of K^+. The reaction was stopped by sudden cooling at 4°C and by the addition of cold perchloric acid (10% final concentration). 0.5 ml of cold charcoal suspension (20%, w/v) was added, and after 5 min the suspension was spun for 3 min at 15,000 × g. The supernatant was analyzed for its ^{32}P content in a liquid scintillation counter. The difference between the incubation in the absence and presence of ouabain was considered to represent the Na^+,K^+-ATPase activity which represented 95–97% of the total activity. The effects of various plasma extracts were expressed as percentages of the ouabain sensitive ATPase.

^3H-Ouabain binding to human erythrocytes was performed as previously described[11], except that the binding was made with 2×10^{-9}M ^3H-ouabain (32 Ci/mmol, Amersham) alone or in the presence of increasing amounts of either unlabeled ouabain or plasma extracts.

The procedure for measurement of digoxin immunoreactivity was essentially that described by Smith et al.[15] using a kit from Amersham.

It was previously reported that the endogenous Na^+,K^+-ATPase inhibitor was heat-stable. Different samples of fresh plasma (from blood bank) were separated immediately from the cells and pooled, then boiled for 20 min. The clot was disrupted and centrifuged at 4°C for 20 min at 50,000 × g. Following centrifugation, 150 ml of the supernatant was applied to a column (5 × 95 cm) of AcA 54 ultrogel (IBF, Villeneuve-la-Garenne, France). The permeation was run at 4°C by 50 mM ammonium acetate pH 7.0 containing 1% of ethanol, at a flow rate of 125 ml/h. Fractions were collected, freeze-dried, reconstituted with $1/_{10}$ of initial volume of 10 mM Tris-HCl pH 7.4 containing 1% of ethanol, and tested. The samples from the previous step were applied to a column (1.6 × 30 cm) of DEAE-cellulose (Cellex D from Bio Rad) which was previously equilibrated at 4°C with 10 mM Tris-HCl pH 7.4 containing 1% of ethanol. The column was washed by 2 column volumes of the equilibrating buffer, before starting the elution gradient. This latter consisted of linearly increasing concentrations of ammonium acetate, from 0 to 0.5 M, in the equilibrat-

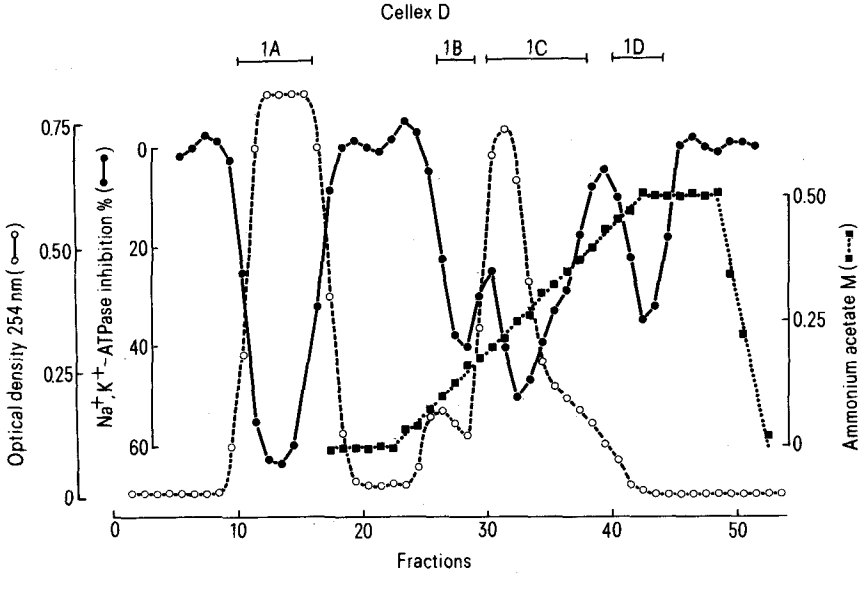

Figure 1. Anion exchange chromatography. The plasma extract 1 from gel filtration was applied to a DEAE-cellulose column (1.6 × 30 cm). The elution was performed at 4°C at a flow rate of 80 ml/h using a linear gradient of 0 to 0.5 M ammonium acetate ■···■, in 10 mM Tris-HCl, 1% ethanol pH 7.4; o——, optical density, ●——, inhibition of the Na^+,K^+-ATPase activity.

ing buffer. The chromatography was run at 4 °C at a flow rate of 80 ml/h. Fractions were collected, freeze-dried, reconstituted by $1/10$ of the initial volume of bidistilled water and tested. The degree of purification was estimated by comparison of the optical density at 254 nm of plasma extracts at a concentration which gives 50% of the maximal inhibitory effect on Na^+,K^+-ATPase to that of the original supernatant of boiled plasma. Three sets of experiments were run on three plasma pool samples.

The fluxes mediated by the Na^+-pump and the Na^+,K^+-cotransport system were measured in fresh red cells as previously described using selective inhibitors[16]. Ouabain blocks the exchange of internal Na^+ for external K^+ catalyzed by the Na^+,K^+-pump. Bumetanide blocks the 1:1 Na^+:K^+ efflux catalyzed by the Na^+,K^+-cotransport system. The ouabain- and bumetanied-resistant Na^+ and K^+ efflux represent the basal membrane 'leaks' for monovalent cations. Li^+,Na^+-countertransport was assessed as the Na^+-stimulated Li^+ efflux by the method previously described[17].

Results and discussion. Gel filtration of the supernatant of boiled plasma produces 1 peak capable of inhibiting Na^+,K^+-ATPase activity. This peak (named plasma extract 1) appearing just after the salt peak is slightly adsorbed onto the gel, thus complicating the estimation of its mol. weight. This agrees with most of the data hitherto published[12,18,19]. The plasma extract 1 is resolved by anion exchange chromatography into 4 peaks inhibiting Na^+,K^+-ATPase activity (fig. 1). The first one (peak 1A) is not retained by the cellex D and corresponds to the plasma cations, and in particular to calcium which is known to inhibit the Na^+,K^+-ATPase activity[20]. The linear gradient eluted three peaks off the cellex D, inhibiting the Na^+,K^+-ATPase activity. These are designated 1B, 1C, and 1D, and were eluted by 150 mM, 250 mM, and 500 mM ammonium acetate, respectively. The last peak (1D) appears partially contaminated by an artifact of the column, as may be occasionally observed with a sample of 154 mM NaCl alone. The calcium concentration of these three peaks is undetectable. The purification coefficient was calculated to be 110, 390 and 250 for the plasma extracts 1B, 1C and 1D, respectively. Therefore, only 2 peaks (1B and 1C) appear to have a specific inhibitory activity as previously suggested[12]. Licht et al. have indicated that plasma contains inhibitors which are excluded from cationic resins[18]. Our present data indicating anionic properties of Na^+,K^+-ATPase inhibitors are consistent with this previous work.

The cross-reaction of the various plasma extracts with the specific antidigoxin antibodies was assessed. All the extracts were devoid of such activity. No clear explanation was found for the discrepancy between our data and the digoxin immunological properties of Na^+,K^+-ATPase inhibitors previously reported[12]. The plasma extracts capable of inhibiting Na^+,K^+-ATPase activity were also found to inhibit the total Na^+ efflux from erythrocytes (fig. 2). When referred to the initial plasma volume, the maximal effect is seen with fractions 1C. The various Na^+ transport systems were subsequently studied with extracts corresponding to 10 ml of initial plasma volume.

The ouabain sensitive Na^+ efflux was inhibited by fractions 1C and 1D (fig. 3A). Three measurements on each set of extracts showed a maximal inhibition ranging from 60 to 80% for fraction 1C. No significant effect could be observed with the 1B fraction at the above concentration. However, at a higher concentration, a significant inhibition by 1B could be observed. 1B, 1C and 1D extracts markedly inhibited the Na^+ efflux

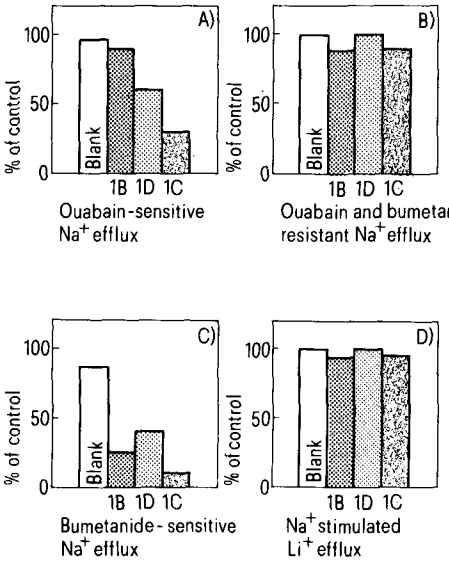

Figure 3. The effect of different plasma extracts (10 ml of initial plasma volume) on different Na^+ transport pathways. *A* The Na^+, K^+-pump. *B* The Na^+ passive permeability. *C* the Na^+,K^+-cotransport. *D* The Na^+,Na^+-countertransport. This figure represents the mean of 3 determinations on each 2 sets of plasma extracts preparations. The inter-assay variation is 15%.

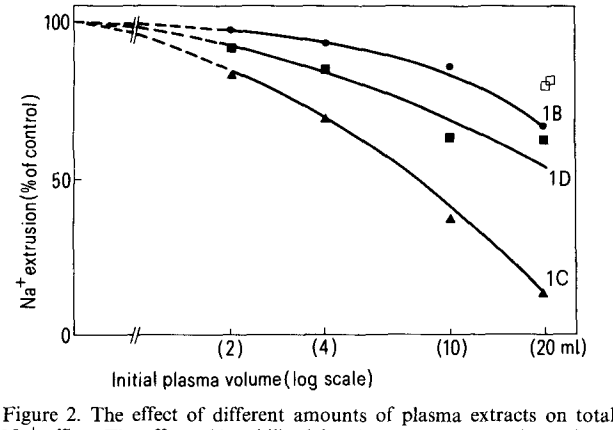

Figure 2. The effect of different amounts of plasma extracts on total Na^+ efflux. The effect of lyophilized fractions were expressed as a function of initial plasma volume. □ represent the effect of fractions 20–22 (fig. 1) of the anion exchange column.

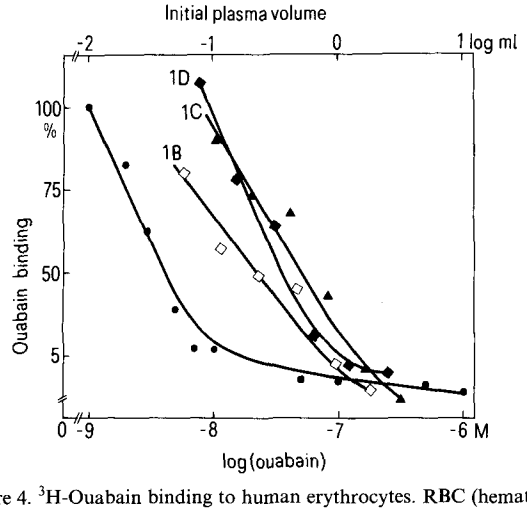

Figure 4. ^3H-Ouabain binding to human erythrocytes. RBC (hematocrit ranging from 1 to 3%) were incubated for 5 h at 37 °C with 2×10^{-9} M of ^3H-ouabain, with increasing amounts of either unlabeled ouabain (●——), or plasma extracts 1B (◇——), 1C (▲——) and 1D (◆——).

catalyzed by the Na^+,K^+-cotransport (fig. 3C). This effect was observed in 4 experiments on 2 sets of plasma extracts. A maximal inhibitory effect ranging from 65 to 95% was observed with the 1B and 1C fractions. No effect of any of the 1B, 1C or 1D fractions could be observed on the ouabain and bumetanide-resistant Na^+ efflux (fig. 3B) or on the Na^+,Li^+-countertransport (fig. 3D). The plasma extracts which inhibit the Na^+,K^+-ATPase activity possess the properties of endogenous sodium transport inhibitor(s) (ESTI). These ESTI decrease 2 Na^+ transport pathways in human red blood cells; the Na^+,K^+-pump and the Na^+,K^+-cotransport, whereas the passive permeability for Na^+ and the Na^+,Na^+-countertransport remained insensitive to ESTI. There is strong evidence suggesting that the Na^+,K^+-pump and the cotransport system represent different molecular entities[21], with preferential affinities for various inhibitors. The Na^+,K^+-pump is selectively inhibited by ouabain and other digitalis-like compounds[22], and the Na^+,K^+-cotransport by furosemide, ethacrynic acid and other loop diuretics[23]. This could lead to the hypothesis that the plasma may contain molecularly different inhibitors of the 2 Na^+ transport systems.

The effect of plasma extracts on specific ^3H-ouabain binding to human erythrocytes is shown in figure 4. The displacement curve obtained with increasing amounts of plasma extract is dose-dependent and similar to that obtained with increasing concentrations of unlabelled ouabain. The amounts of plasma extract 1B, 1C and 1D which displace the ^3H-ouabain binding by 50% correspond to 300 µl, 660 µl and 520 µl of initial plasma volume, respectively. Such a displacement is obtained by 2×10^{-9} M unlabeled ouabain. The effects of plasma extracts 1 and 1A were not assessed on either ouabain binding or Na^+ fluxes, as they have a high cationic content. The anion plasma vanadate, which is known to inhibit Na^+,K^+-ATPase activity could be eluted off the cellex D. However, the extracts 1B, 1C, and 1D, in addition to inhibiting the enzyme activity, also displace ^3H-ouabain from its binding sites, whereas vanadate up to 10^{-5} M is devoid of this latter effect. Thus, vanadate cannot fully account for the described effects.

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2 To whom correspondence should be addressed.
3 Kramer, H.J., and Gonick, H.C., Nephron *12* (1974) 281.
4 Bourgoignie, J.J., Klahr, S., and Bricker, N.S., J. clin. Invest. *50* (1971) 303.
5 Sewell, R.B., Hughes, R.D., Poston, L., Williams, R., Clin. Sci. *83* (1982) 237.
6 Hamlyn, J.M., Ringel, R., Schaeffer, J., Levinson, P.D., Hamilton, B.P., Kowarski, A.A., and Blaustein, M.P., Nature *300* (1982) 650.
7 Mac Gregor, G.A., Fenton, S., Alaghband-Zadeh, J., Markandu, N., Roulston, E., and Wardener, H.E., Br. med. J. *283* (1981) 1355.
8 Haddy, F.J., and Overbeck, H.W., Life Sci. *19* (1981) 1355.
9 Blaustein, M.P., Am. J. Physiol. *232* (1977) C165.
10 De Wardener, H.E. and Mac Gregor, G.A., Kidney Int. *18* (1980) 1.
11 Cloix, J.F., Devynck, M.A., Elghozi, J.L., Kamal, L.A., Lacerda-Jacomini, L.C., Meyer, P., Pernollet, M.G., Rosenfeld, J.B., and De Thé, H., J. Hypertension (1983) in press.
12 Gruber, K.A., Whitaker, J.M., and Buckalew, V.M., Nature *287* (1980) 743.
13 Clarkson, E.D., Raw, S.M., and De Wardener, H.E., Kidney Int. *16* (1979) 710.
14 Sealey, J.E., Kirshman, J.D., and Laragh, J.H., J. clin. Invest. *48* (1969) 2210.
15 Smith, T.W., Butler, V.P., and Haber, E., N. Engl. J. Med. *281* (1969) 1212.
16 Dagher, G., and Garay, R.P., Can. J. Biochem. *58* (1980) 1069.
17 Canessa, M., Adragna, N., Solomon, M., Connolly, T.M., and Tosteson, D.C., N. Engl. J. Med. *302* (1980) 772.
18 Licht, A., Stein, S., McGregor, C.W., Bourgoignie, J.J., and Bricker, N.S., Kidney Int. *21* (1982) 339.
19 Lichtstein, D., and Samuelov, S., Proc. natl Acad. Sci. USA *79* (1982) 1453.
20 Lindenmayer, G.E., Pharmac. Theor. *2* (1976) 843.
21 Karlish, S.A., Ellory, J.C., and Lew, V.L., Biochim. biophys. Acta *646* (1981) 353.
22 Glynn, I., and Karlish, S., A. Rev. Physiol. *37* (1975) 13.
23 Ellory, J.C., and Stewart, G.W., Br. J. Pharmac. *75* (1982) 183.

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Photoregulation of some enzymes from *Neurospora crassa*

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Summary. Light-grown cultures of *Neurospora crassa* showed photoregulation of a number of enzymes. Proteases and cytosolic malate dehydrogenase showed an increase in activity. There was a decrease in the activity of mitochondrial malate dehydrogenase, isocitrate dehydrogenase and cytosolic glucose-6P-dehydrogenase, isocitrate dehydrogenase and isocitrate lyase.
Key words. Neurospora crassa; enzyme photoregulation; cultures, light-grown; photoregulation, enzyme; carbohydrate metabolism.

Photoregulation of both metabolic processes and development has been shown to play an important role in the life of plants. One of the most striking examples is that of the obligatory light dependent chlorophyll formation in angiosperms[1]. Photoregulation of carotenoid biosynthesis has also been reported for angiosperms, algae, fungi and bacteria and has been extensively reviewed[2-6]. Information is lacking regarding the photoregulation of primary metabolism in fungi. Attempts have been made in the present studies to understand the influence of light on carbohydrate metabolism in *Neurospora crassa*. Light mediated changes in primary metabolism may subsequently help us to understand the regulation of secondary metabolism, thereby leading to an increase in the production of secondary metabolites.

Materials and methods. The carotenogenic strain of *N. crassa* (wild type) obtained from the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India, was maintained on Saboraud's agar slants. The synthet-