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Discordant findings concerning the stimulation by chlorpromazine of erythrocyte sugar transfer

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It is commonly observed that chlorpromazine and other psychoactive phenothiazines suppress permeation or transport mechanisms in biological membranes. The obvious potential relevance to the clinical applications of these drugs has focused attention particularly on their interference with the uptake of the prominent biogenic amines in brain slices [1–4] and in synaptosomes [3–9]. But similar blocking by chlorpromazine is evident also not only in the non-neural traffic in amino compounds [1, 10–15], but in a wide assortment of membrane transports, dealing with the active turnover of Na^+ and K^+ [16–19], the accumulation of Ca^{2+} within sarcotubular vesicles and other organelles [20–23], and the uptake of glucose and other simple sugars by various mammalian cells [19, 24–27] and even by protozoa [28].

However, one series of studies indicates that the action of chlorpromazine and related agents on sugar transport can be strongly stimulatory, rather than inhibitory. Examining the effects of such drugs on the facilitated diffusion of hexoses through human red-cell membranes, Baker and Rogers [29–32] found a sharply biphasic or triphasic response, as a function of the concentrations applied. Indeed, their dose–response curves show a virtual discontinuity at a critical chlorpromazine concentration range, where the general pattern of progressive transport inhibition is interrupted by an abrupt and marked acceleration of the net transfer of glucose or sorbose. In contrast, their parallel isotopic-tracer studies revealed no significant response to chlorpromazine in the equilibrium exchange of glucose, either at the critical levels where net entry and net exit were stimulated or (as confirmed by Morais *et al.* [27]) at the higher levels which distinctly blocked net movements.

Analysis of the operation of this mechanism that mediates the equilibration of monosaccharides across human erythrocyte membranes had been a central concern in our laboratory for many years, and we were hopeful of utilizing the unusual behavior of chlorpromazine described by Baker and Rogers, as a tool for improving differential radiochemical labeling of the membrane components involved in the transport. The feature that particularly engaged our attention was the marked enhancement of net sugar transport inducible with this drug (a response not seen in this insulin-independent system with any of the several other identified classes of inhibitors). However, we have been quite unable to confirm this critical phenomenon with chlorpromazine, using either of the two principal test procedures adopted by Baker and Rogers.

The studies reported here were pursued in three episodes: first (by D. M. S., as a student pilot project) throughout the academic year 1975–76, then (by P. G. LeF.) for several weeks in late 1977 and again in early 1980. Similar results were obtained on each occasion.

Methods. Human blood, either heparinized or in standard acid–citrate–dextrose bags, was obtained from the Nassau–Suffolk (N.Y.) Inter-County Blood Services, and stored at about 3°. A few hours prior to experimental work, the plasma and additives, together with the major part of the white cells, were removed by several serial centrifugal washes, first with isotonic saline solution (sometimes lightly citrated) until clotting factors were sufficiently diluted, and then with a balanced-salt medium.

Duplication of the nominal essentials of Baker and Rogers' principal experiments required only minor procedural alterations in techniques employed extensively in our laboratory for many years. For the densitometric (Ørskov) recording of D-glucose exit, the erythrocytes were suspended at approximately 5% (v/v) in the medium, with D-glucose at 75–150 mM, and incubated at 37.5° for 50–80 min, to assure equilibration of the sugar throughout the cell water. The suspensions were then centrifugally concentrated to varying degrees as high as 64% (v/v), to allow adequate dilution of the sugar upon reduction of the cell density to the level of about 0.3% required in the cuvette for satisfactory Ørskov densitometric recording [33]. The initial volume in the cuvette (continuously stirred at 37.5°) was usually either 12 or 13 ml, and contained the chosen concentrations of chlorpromazine and glucose. Recording was begun at the instant when glucose exit was initiated by injection of a fixed volume (varying on different occasions from 60 μl up to 1 ml) of the cell suspension. "Exit times" were estimated directly from the records as described by Sen and Widdas [34]. On a few occasions (noted below), the assigned levels of chlorpromazine were added also to the glucose-loaded cells, permitting preincubation with the drug prior to the start of sugar exit.

The isotopic tracing of L-sorbose uptake requires denser cell suspensions, in which a significant fraction of the drug is removed by the cells [35], subtracting to a variable degree from the nominal concentrations applied. For this reason, aliquots of the cell preparations were taken through three initial centrifugal washes in approximately 100-fold volumes of medium containing the several assigned chlorpromazine concentrations, and each was then brought to a fixed volume at approximately normal blood hematocrit. A small volume (generally 50 μl) from one of these dense suspensions was taken for each sorbose-uptake assay (run at 37–38°). Uptake was initiated by addition of an equal

volume of medium containing about 25 nCi of uniformly labeled [^{14}C]-L-sorbose at a total sorbose level of 150 mM, plus the same chlorpromazine concentration already surrounding the cells. After 60 sec of incubation, the sugar uptake (still in its early, nearly linear phase) was terminated by removal of 50 μl of the mixture into a much larger volume of ice-cold hypertonic saline containing 2 mM $HgCl_2$ (a conventional "quencher" for this transport system), followed immediately by either filtration through a 0.22 μm Millipore filter (GSWP) or rapid centrifugal separation and draining of the cells. Cell radioactivity was estimated by automatic liquid scintillation counting (Picker Liquimat 330 or Nuclear-Chicago Mark II) in Aquasol 2, using either the Millipore filters directly (after allowing a burst of chemiluminescence to subside) or Somogyi filtrates [36] of the sedimented cell pellets. The comparative net uptakes were calculated by subtraction of the average recovery in parallel preparations that had been mixed with the "quencher" prior to addition of the labeled sugar.

Most of the work was carried out in a balanced-salt medium which for many years has been our standard vehicle for handling mammalian cells *in vitro* [37]. This is a mixture of the chlorides of Na^+, K^+, Ca^{2+}, Mg^{2+}, and $Tris^+$, respectively, at 118, 4.8, 2.6, 1.7, and 35 mM, pH 7.4, with a total tonicity of 300 ± 3 mOsM. The dissonance of our early findings suggested testing of the significance in the inclusion of Mg^{2+} and the substantial Tris buffering, since Baker and Rogers identify their medium [29] as including only Na^+, K^+, Ca^{2+}, Cl^-, and HCO_3^-, respectively, at 155.8, 5.6, 4.3, 163.9, and 1.8 mM. But for proper electrical balance of this formula, one must presume the actual Ca^{2+} content per liter to have been 4.3 m-equiv. rather than 4.3 mmoles; and in switching to the Baker–Rogers medium for about a third of the experiments during each phase of these investigations, we have generally followed this presumption. For several additional specific tests, this level was doubled, or the $CaCl_2$ was omitted altogether. Also, as might be anticipated, only very loose buffering is provided by the 1.8 mM bicarbonate (nominally establishing pH 7.2) in this mixture. We found the freshly prepared medium to be near pH 7.8, with a shift to as low as pH 6.8 upon addition of any substantial proportion of saline-washed red cells. However, none of these variations in the medium appeared to have any bearing on the dissonance between the observations in the two laboratories.

All sugar solutions were either made fresh on the day of use, or thawed from stocks stored frozen for periods of up to 2 weeks. A fresh stock solution of the chlorpromazine (at 40–400 μM) was prepared in the experimental medium for each day's work. The reagent used was Smith Kline & French's crystalline hydrochloride. Since the initial sample (a gift from the late Dr. George C. Cotzias of Brookhaven National Laboratory) dated from approximately 1973, a second, fresh preparation was donated directly by Smith, Kline & French Laboratories in early 1976. But no differences in either qualitative behavior or potency between the older and the fresh reagents could be detected at that time, nor during the subsequent reinvestigations.

Results and discussion. Baker and Rogers [29] defined a rather narrow range of chlorpromazine concentrations which greatly augmented the net exodus of glucose that ensues upon placing preloaded erythrocytes in a sugar-poor medium at approximately body temperature. The left-hand panel of Fig. 1 reproduces the data from the one published experimental illustration of this type. A small inhibitory effect is apparent with the drug at 10 μM; but by 15 μM the rate of sugar exit rises to three to four times the control rate. Beyond this critical range, progressive inhibition again dominates the picture.

In the course of many attempts on our part to reproduce these experiments by closely scanning around this concentration range, no such discontinuity in the dose–response curve has ever appeared. The data of the right-hand panel of Fig. 1 typify our findings. A slight transport acceleration at the lower chlorpromazine levels was seen when the external sugar concentrations were relatively high, but at most this amounted to about 20%. The scatter in a typical series of such measurements, distributed over the critical range of chlorpromazine concentrations, is given in Table 1. Motais *et al.* [27] also noted little deviation from control exit rates for glucose (at 27°) in the presence of 10 μM chlorpromazine.

Other differences between our observations on glucose exit and those of Baker and Rogers are of a minor quantitative nature: (1) we find appreciably higher control rates—more so than can be attributed to the slightly lower temperature (36°) used for their studies; and (2) we find a somewhat lesser sensitivity to the inhibitory action of the chlorpromazine. We are in full agreement, however, in noting (1) no significant increase in the apparent half-saturation level for glucose transport until the drug levels approached 100 μM, and (2) no alteration of the results when the cells were pretreated with the drug (in lieu of introducing it only at the "zero time" of glucose egress).

For net hexose transfer in the reverse direction (uptake into the cells), the discontinuities observed by Baker and Rogers in the dose–response curve for chlorpromazine were even more distinct, because under these circumstances a more prominent inhibition was seen at concentrations below the critical range [31, 32]. The accelerative phase might then not suffice to bring the transport rate above the control figure, but imposed a sharp three-phase pattern on

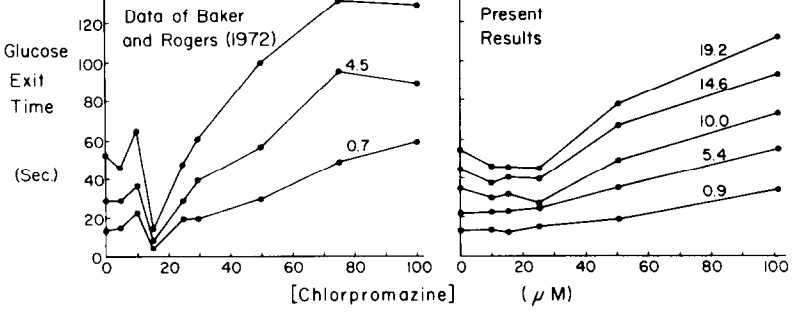

Fig. 1. Conflicting findings on the effects of chlorpromazine on the exit of D-glucose from human erythrocytes, as recorded by Ørskov densitometry. Numerals above curves are external glucose millimolarities. Data at left are redrawn from Baker and Rogers [29] (36°, cells preloaded at 76 mM); present data at right are from one typical parallel experiment (37.5°, same medium, cells preloaded at 105 mM).

Table 1. Very limited response of erythrocyte net sugar transport to chlorpromazine in critical concentration ranges defined by Baker and Rogers [29]*

[Chlorpromazine] range (μM)	Exit-time ratio (treated/control) [Average ± S.D. (N)]
2–5	0.994 ± 0.037 (8)
5–8	0.963 ± 0.048 (7)
8–12	0.978 ± 0.058 (9)
12–16	0.959 ± 0.067 (8)
16–20	1.072 ± 0.053 (4)

* D-Glucose exit measurements carried out as for Fig. 1, but in Tris-buffered medium defined in Methods.

the curve, as illustrated for L-sorbose uptake in the upper set of data in Fig. 2. (Even for glucose exit, Baker and Rogers report a similar picture when the temperature is lowered to 17°, since the critical drug level is then raised somewhat [31].)

Here again, however, none of our tests confirmed the discontinuity in the response pattern. The lower records of Fig. 2 show the results from two such experiments with L-sorbose entry in our hands, nominally reproducing the situation defined for the accompanying data of Baker and Rogers [31, 32]. Only a minor acceleration, of borderline resolvability, may be indicated for the lowest chlorpromazine level tested (5 μM); and the gradually increasing inhibition at higher levels is here only slightly greater than in our studies of glucose exit (Fig. 1). A very similar pattern of response has been reported for glucose transport through red-cell ghost membranes by Matus *et al.* [38], except that the sensitivity to the drug was substantially lower than in the present case.

As noted in Methods, we have been unable to ascribe these conflicts to any differences in the composition of the media used. Personal communication (with Dr. G. F. Baker, 1976) also failed to identify any other experimental considerations as likely factors in the problem. One distant possibility that was suggested concerned the packaging of the chlorpromazine hydrochloride reagent used by Baker and Rogers; their stock solutions were diluted from commercial ampoule preparations containing small amounts of citrate, metabisulfite, and sulfite. However, addition of these ingredients to fresh chlorpromazine solutions, in the ratios specified for these ampoules, produced no discernible change in the behavior of the drug in these experiments; and the system was not overtly affected even by 100-fold higher levels of these additives in the absence of the chlorpromazine.

Our data have not suggested any dependency of the erythrocyte response on the duration of storage of the blood; but an editorial referee's inquiry has spurred us recently to check specifically the behaviour of unstored cells in this situation. The responses of glucose exit (from initial cell levels near 100 mM, into external levels of 7–10 mM) to various doses of chlorpromazine were recorded densitometrically on two batches of cells prepared immediately upon drawing of the blood samples (from the investigator). However, the dose–response patterns observed in these very fresh preparations did not detectably deviate from those derived from blood-bank sources, such as represented in the right-hand panel of Fig. 1.

In summary, repeated reinvestigation over a period of years has failed to confirm the reported discontinuity in the dose–response characteristics for chlorpromazine action on net hexose transfer through human red-cell membranes.

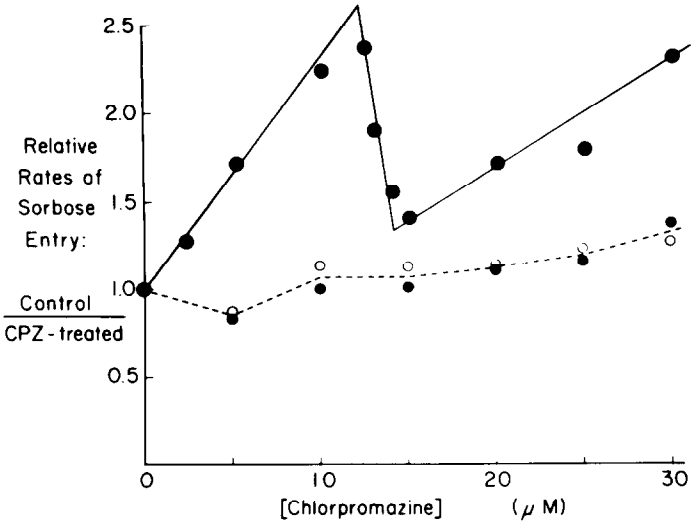

Fig. 2. Conflicting findings on the effects of chlorpromazine on the entry of L-sorbose into human erythrocytes, as traced by isotopic methods. Data of upper record are redrawn from Baker and Rogers [31, 32] (36°, sorbose concentration unspecified); present data (open and filled points defining lower curve) are from two typical parallel experiments (37–38°, Tris-buffered medium, external [sorbose] initially about 90 mM).

Both Ørskov optical densitometric recording of D-glucose exit and direct isotopic tracing of L-sorbose entry show in our hands only a slight acceleration of sugar transport at sub-inhibitory concentrations (around 10^{-5} M). We are unable to account for this direct dissonance with the findings of Baker and Rogers [29–32].

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