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6. There were five treatment groups: (i) 14 animals received HRP in saline and served as controls; (ii) 23 received HRP in DMSO; (iii) 12 received 0.5 to 1 ml of DMSO intraperitoneally plus 0.25 ml of HRP in saline intravenously; (iv) 24 received 0.5 ml of 10 to 30 percent DMSO intraperitoneally plus 0.25 ml of HRP in 10 to 30 percent DMSO intravenously; (v) 7 received 0.5 ml of 15 percent DMSO intraperitoneally plus 0.25 ml of 15 percent DMSO intravenously, and 2 to 24 hours later, HRP in saline intravenously.
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14. Additional studies in our laboratory are being conducted on larger animals to determine if DMSO does indeed exert an effect on tight junctions. In these animals HRP in DMSO is injected into the femoral vein, and carotid blood is sampled for its osmolarity. Because the DMSO effect on the barrier appears to be reversed within 2 hours, opening of the blood-brain barrier has been looked for in mice in which DMSO/HRP has been permitted to circulate through the brain for 1½ to 5 minutes. At 5 minutes, numerous focal exudates of HRP surround blood vessels throughout the brain. These sites are currently being viewed ultrastructurally to determine if the leaks are attributed to open tight junctions or vesicular transport. In other animals DMSO is injected into the lateral cerebral ventricle and HRP is administered intravenously to determine whether the DMSO effect on the abluminal surface of the blood-brain barrier is similar to that observed on the luminal surface.
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13 April 1982

## Boradeption: A New Procedure for Transferring Water-Insoluble Agents Across Cell Membranes

Abstract. *A new process has been developed which is called "Boradeption" to signify boronic acid–dependent phase transfer of water-insoluble agents. Highly fluorescent boronic acid derivatives, FluoroBoras, are solubilized with a physiologically compatible carrier buffer containing a receptor group for boronate adduct formation. The system can be used to stain living cells. In another variation of the Boradeption concept, an insoluble reporter molecule containing a boronate receptor is solubilized with a carrier buffer containing a boronic acid functional group. The boronate-receptor complexes, which are in dynamic equilibrium, can be designed as vital stains and reagents for a variety of biological and medical applications.*

We have developed new reagents and procedures for transferring water-insoluble agents across membranes into living cells. The principles involved are applicable to phase transfer in a variety of biomedically and chemically significant situations. We call the process "Boradeption" to indicate boronic acid–dependent phase transfer.

Aryl and alkyl boronic acids react with a variety of compounds having amino and hydroxyl groups and form five- or six-membered cyclic complexes of varying stability. The ability of such complexes to resist hydrolysis depends on steric factors and ring size, such that the boronate adducts are either triagonal or tetragonal complexes. This is related to the effective Lewis acid strength of the boronic acid at the *p*H of the buffers employed.

We coupled certain reporter groups to boronic acids to form ReportaBoras. Starting with a 1,3-diaryl-$\Delta^2$-pyrazoline [useful as an optical brightener (*1*)], 1-(phenyl-*p*-sulfonic acid),3-phenyl-$\Delta^2$-pyrazoline sulfonyl chloride was coupled to *m*-aminophenylboronic acid (*2*). This highly fluorescent pyrazoline-sulfamido-phenylboronic acid is very insoluble in water and thus would seem to be useless as a stain in living tissue. However, a variety of carrying buffers with receptor groups able to form complexes effectively with boronic acids can be used to solubilize this and other insoluble boronic acids at physiological *p*H values (see scheme 1 in Fig. 1A).

The extent of the dynamic equilibria in water between the complexing carrying buffer containing the receptor for the boronic acid and the normally water-insoluble reportabora can be used to great advantage to carry the latter into aqueous solution as a complex, then as the free compound through hydrophobic zones on the cell membranes, and finally into cellular components. In living cells, the transferred reportabora can be dynamically distributed into both hydrophobic zones and into areas where relatively tight boronate complexes can be formed. One can vary the hydrophobicity and net charge of the reporter group, the *p*K and Lewis acid complexing strength of the selected boronic acid, and the complexing potential of the carrying buffer so that selective cellular uptake, transport, and binding of the reporter in the cell give staining patterns reflecting important structural and metabolic cell components. Among the reporter groups can be various fluorophores (FluoroBoras), chromophores (ChromoBoras), heavy metal compounds (MetalloBoras), and drugs (TheraBoras). Zwitterion buffers that contain appropriate hydroxyl and amine functions (*3*) are particularly useful as boronate-carrying buffers. Such buffers are generally nontoxic to cells, and those which also contain sulfonates are generally not taken up by cells.

In a variation of scheme 1, it is possible by scheme 2 to design reporter groups attached to boronate receptors (reportareceptors) that react with boronic acids, and then to use as carrying agents or buffers various soluble boronic acids (carrierboras) (Fig. 1B). For example, one attaches *N*-(3-aminopropyl)-diethanolamine to various reporter agents and then carries these reportareceptor compounds as soluble complexes of certain aryl sulfonic acids containing a boronic acid.

Certain FluoroBoras, such as *m*-dansylamidophenylboronic acid (FBI) and *m*-darpsylamidophenylboronic acid (FBII), have the additional valuable feature of extensive enhancement of their fluorescence when placed in hydrophobic regions, as in certain proteins. There usually is a shift in fluorescent emission wavelength.

The new boronate adducts that form with the cellular components can have a wide range of stabilities by virtue of the nature of the complexing cellular receptor components and the relative lack of a competing hydrolytic solvent, such as water, in various hydrophobic cellular regions. By manipulation of (i) the charge and other chemical features of the


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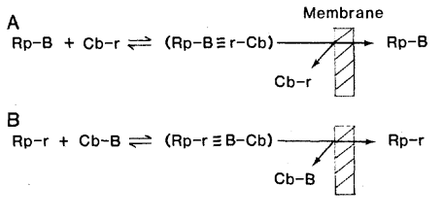


Fig. 1. Schematic representations of the dynamic equilibria and extraction into cells of reporter molecules. (A) Scheme 1. A normally water-insoluble reportabora (*RP-B*) is solubilized as an equilibrium complex by reaction with an excess of a carrier buffer containing a receptor group for boronate-adduct formation (*Cb-r*). In the presence of a cell membrane, the equilibrium of adduct formation is displaced such that the free reportabora can now enter the lipophilic portions of the cell. (B) Scheme 2. A normally water-insoluble reportareceptor (*Rp-r*) is solubilized as an equilibrium complex by reaction with an excess of a carrier buffer that contains a boronic acid capable of forming an adduct with the reportareceptor. In the presence of the cell membrane, the adduct equilibrium is displaced such that the free reportareceptor can enter the cell.

reporter group and (ii) the complexing strength of both the boronic acid and the carrying buffer, additional specificity can be added to the staining process. Furthermore, fluorescent boronic acids can be prepared in which the fluorophore is separated from the acid by a substrate for a specific enzyme—perhaps a protease, esterase, or glycosidase—in such a manner that cleavage of this substrate releases the boronate from the fluorophore in the cell, altering the staining pattern from one that would be obtained with a related but noncleavable mock FluoroBora substrate.

In scheme 2, reporter groups attached to various boronate receptors (such as diethanolamines, polyamines, and cyclic nitrogen or oxygen crown-like compounds), can also be transferred into cells. The distribution of such reportareceptors can also be of considerable utility.

In appropriate buffer solutions, certain complexes between FluoroBoras and carrying buffers can be sufficiently stable that the FluoroBora component will not be removed from its carrying buffer and thus not be fully transferred into organic solvent areas or hydrophobic regions. Thus the fluorescence of some complexes may not be fully enhanced in the presence of proteins such as serum albumin. However, subsequent cleavage of the boronate or receptor groups frees the fluorophore from the carrier, releasing the now relatively insoluble fluorophore. It can then pass into an organic zone or into a hydrophobic pocket of certain proteins, becoming more fully enhanced in fluorescence, thereby reporting the cleavage event. Such behavior is seen with FBI and FBII carried by TAPSO buffer; other FluoroBoras can be prepared in which substrate groups are placed between the reporter group and the boronate (scheme 1) or boronate receptor (scheme 2) such that alteration or cleavage of these groups by enzymes or their reaction products can release modified fluorophores from the solubilizing complexes, which enter hydrophobic pockets. Fluorescent enhancement then occurs, leading to sensitive and facile measurement of the overall reaction. For example, addition of $H_2O_2$ causes cleavage of the carbon-boron bond in FBI to form *m*-dansylamidophenol, which, no longer held by the carrying buffer, passes into serum albumin pockets, where its fluorescence is fully enhanced.

We compared complexes of the three FluoroBoras [FBI, FBII, and 7-methoxycoumarin-4-methylene oxycarbonylphenyl-3′-boronic acid (FBIII)] and various carrying buffers in the ability of serum albumin to enhance or quench fluorescence of the FluoroBora component of the complex and in the extractability by ethyl acetate of the FluoroBora component. We found that 3-[*N*-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TAPSO) forms the most stable boronate complexes and 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid (MOPSO) the weakest. All the FluoroBoras are maximally extracted from MOPSO (93 to 99 percent) by ethyl acetate, with much less extraction from TAPSO (28 to 62 percent). Consistent with this, the fluorescence of FBI and FBII is enhanced less in TAPSO than in MOPSO. On the other hand, the fluorescence of FBIII in the presence of serum albumin is minimally quenched in TAPSO and maximally quenched in MOPSO, since hydrophobic regions quench fluorescence of the FBII fluorophore. TAPSO keeps these reportabora compounds out of the hydrophobic pocket of serum albumin more effectively than the other carriers. Figure 2 shows one form of such a water-soluble transannular boronate:TAPSO complex.

For the staining procedure, the FluoroBoras were generally dissolved first in 100 μl of dimethylacetamide and then diluted to 20 ml with the physiologically isotonic carrier buffer at *p*H 7.4. The FBI was usually prepared at a concentration of 1 to 1.5 mg in 20 ml. The FBII was considerably more fluorescent and was usually prepared at a concentration of 0.5 to 1 mg in 20 ml. The solutions were

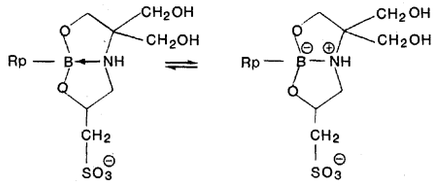


Fig. 2. Diagram showing a water-soluble transannular equilibrium complex between a reportabora and TAPSO, a carrier-receptor. This complex is of the Rp-B≡r-Cb type, where Rp-B is the reportabora and Cb-r is the carrier containing the boronate-receptor (see Fig. 1A).

filtered through a 0.45-μm Micropore filter just prior to use. The FBI solution was usually prepared in 25 m*M* MOPSO containing 140 m*M* NaCl and adjusted to *p*H 7.4 with NaOH. Cells stained with FBI in this buffer show a brighter green fluorescence than in TAPSO, as more dye is accessible to the hydrophobic pockets of the cell. FBII is most effective when used in 25 m*M* TAPSO containing 140 m*M* NaCl and adjusted to *p*H 7.4 with NaOH.

Cells grown on cover slips were washed three times with the appropriate buffer, covered with the FluoroBora solution, and incubated for about 5 minutes at room temperature. The excess reagent was removed by three or four washes with buffer and the cells were mounted upside down in buffer on a glass slide. Observations were made with a Zeiss standard fluorescent microscope with dark-field illumination in ultraviolet light. In order to observe cells stained with FBI or FBII, an exciter filter with a peak transmission at 350 nm was used; in addition, barrier filter 50 or 53 was used for FBI-stained cells and barrier filter 41 for FBII-stained cells. Pictures were taken with a C-35 Zeiss camera on Ektachrome 160 Tungsten film.

Cells in suspension can also be stained by FluoroBoras and then separated by sorting techniques based on the different staining properties of mitotic and interphasic cells (mitotic cells become more fluorescent than do interphasic cells).

After 5 to 15 minutes of exposure to the dyes, the cells were still firmly attached and could be returned to the culture medium and grown without showing signs of toxicity. Cells cultured for up to 4 days grew at approximately the same rate and, after trypsination and seeding, attached with the same efficiency (80 to 90 percent) as untreated cells.

By removing the cells after staining and placing them in isotonic carrier buffers, a dynamic destaining process occurred in which certain regions retained

fluorescent staining for much longer periods of time. Additional valuable information on certain structures and on cell cycle may be obtained by the use of such destaining.

Various protozoa, such as the ciliate *Tetrahymena*, can be stained in vivo and observed for a long period without any apparent decrease in cell motility or signs of cell lysis. In experiments with *Tetrahymena*, a drop of medium containing the organisms was placed on a slide and gently covered with a cover slip. A drop of the FluoroBora solution was then added at a corner of the cover slip. As observed under the fluorescent microscope, the cells became increasingly fluorescent without apparent loss of vi-

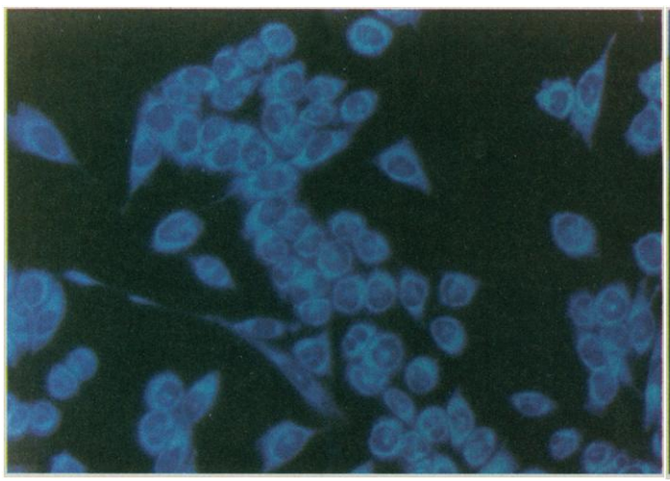

Fig. 3 (left). Asynchronous CHO cells stained with FBII (×160).

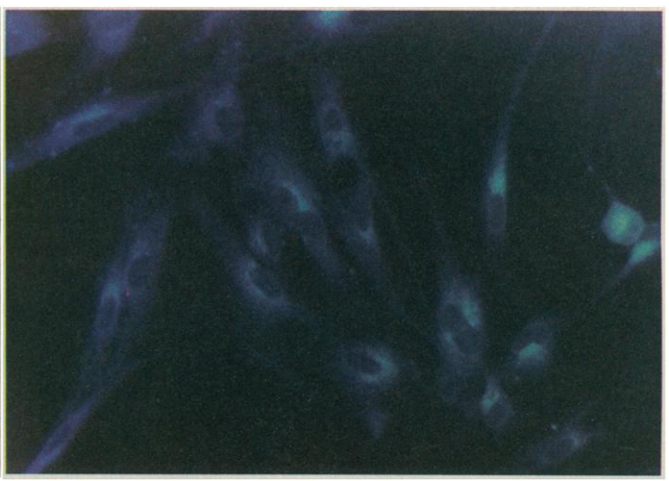

Fig. 4 (right). Fetal human lung fibroblasts (IMR90) stained with FBII (×160).

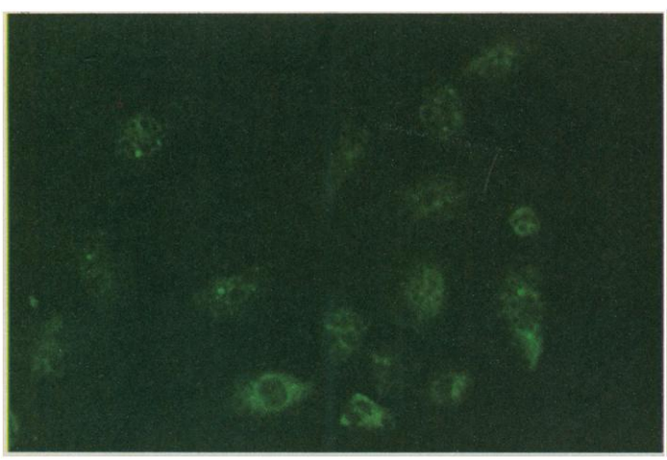

Fig. 5 (left). Synchronized CHO cells in S phase stained with FBI (×160).

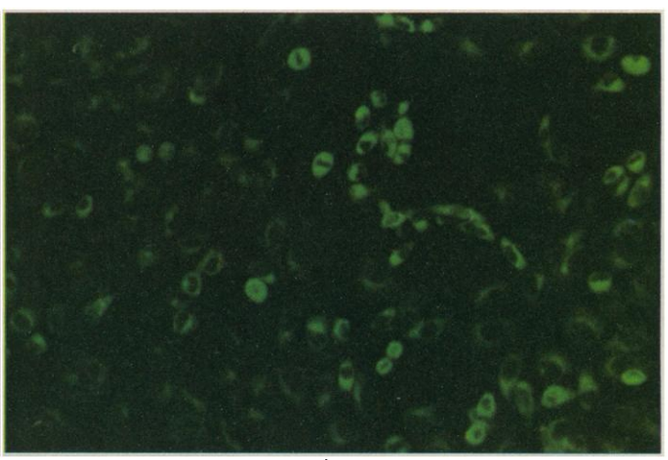

Fig. 6 (right). Asynchronous rat skin fibroblasts (FR3T3) stained with FBI (×160).

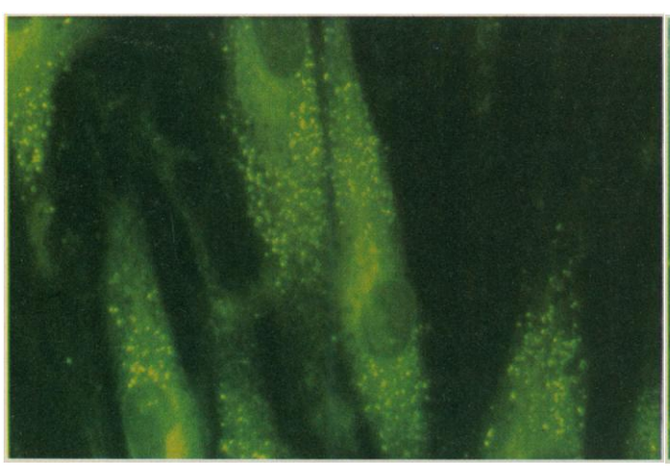

Fig. 7 (left). Cultured human skin fibroblasts from a β-glucuronidase–deficient patient (GM121) stained with FBI (×400).

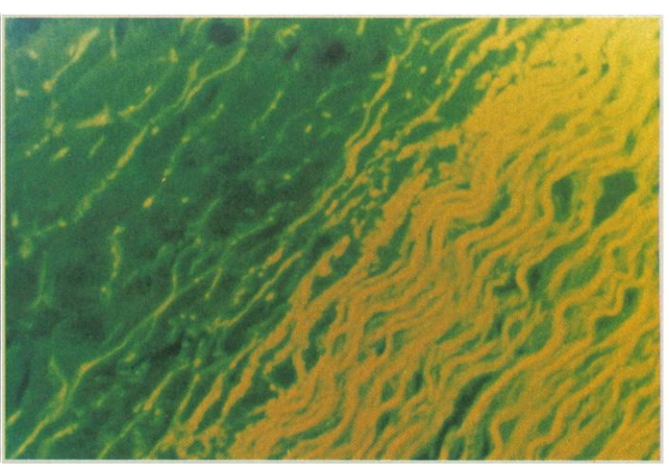

Fig. 8 (right). Section of human aorta stained with FBI. The elastic fibers (bright yellow) are clearly distinguished from other components in the tissue (×160).

tality. In contrast, acridine orange reduces cell motility and causes lysis in a few minutes.

Cells stained with FBII show a strong blue and azure fluorescence, which is intense around the nucleus in interphasic cells and very intense in most of the cytoplasm in mitotic cells. Cells stained with FBI show a strong green fluorescence with a distribution in interphasic and mitotic cells similar to the distribution of the blue fluorescence.

Figure 3 shows asynchronous Chinese hamster ovary (CHO) cells stained with FBII. Although the nuclei generally appear relatively unstained, in some nuclei weak fluorescence is apparent toward the center. Cells in or near division are more intensely stained, suggesting that the rounding of these cells places more hydrophobic regions near the surface. Fetal human lung fibroblasts (IMR 90) stained with FBII are shown in Fig. 4. In these cells the fluorescence is brightest around the nucleus in the Golgi area and the nucleus appears unstained. In the mitotic cells most of the cytoplasm is brightly stained but, again, the fluorescence is more concentrated around the nucleus.

Figure 5 shows synchronized S-phase CHO cells stained with FBI. Particularly striking are the fluorescent spots at the nuclear poles, suggesting that cytoskeletal protein in the centrioles has a high affinity for FBI. Rat skin fibroblasts (FR3T3) stained with FBI are shown in Fig. 6. The brightly fluorescent mitotic cells are clearly visible, and the interphasic cells show brighter fluorescence in the perinuclear areas than in the rest of the cytoplasm.

Cells removed from patients with so-called lysosomal disorders and stained with FBI show unique staining properties reflecting abnormal lysosomal patterns. As an example, cultured human fibroblasts from a β-glucuronidase–deficient patient (GM121) stained with FBI are shown in Fig. 7. Cytoplasmic fluorescent particles (lysosomes?) are evident which are much larger and more numerous than those seen in fibroblasts from normal individuals. The area of the Golgi apparatus, which shows brighter fluorescence than the rest of the cytoplasm, does not contain such particles.

Certain proteins can be easily stained by FluoroBora–Carrier buffer systems or ChromoBora–Carrier buffer systems. For example, serum albumin can be treated with dimethylaminonaptho-azomethoxyphenylsulfamidophenyl - 3 - boronic acid or *p*-dimethylaminophenylazophenylthioureidophenyl - 3 - boronic acid in TAPSO at *p*H 9.0. At this *p*H, much more of the highly insoluble ChromoBoras can be solubilized by TAPSO. As the ChromoBora rapidly enters hydrophobic zones of serum albumin, a short reaction period is followed by dialysis against TAPSO at lower *p*H. The dialysis gradually removes excess ChromoBora without allowing its precipitation from solution, leaving intensely stained protein that cannot be destained except by organic solvents. A protein such as elastin, which is insoluble and highly hydrophobic, can easily be stained by a variety of fluorescent or colored Boradept complexes. The properties of elastin can be employed to prepare a substrate for elastase and other proteolytic enzymes and also to detect elastin in tissue sections (Fig. 8).

Not only insoluble fluorophores and chromophores can be carried into living cells and across the blood-brain barrier by Boradeption, but also modified insoluble drugs, enzyme substrates, heavy metal organic compounds, haptens, and radioactive agents. Past attempts to design effective boron-containing antitumor agents for neutron-capture therapy (*5*) which, on slow neutron bombardment of the boron, release ionizing alpha particles into the tumor with high efficiency, have been relatively unsuccessful. In part, the failures resulted from the limited solubility of most organic boron compounds. These compounds might be taken up by tumor cells if they could be delivered to tissues under physiological conditions. Perhaps this potentially important area of tumor pharmacology should be reexamined in light of some of the present observations.

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## Controlled Drinking by Alcoholics? New Findings and a Reevaluation of a Major Affirmative Study

Abstract. *Controlled drinking has recently become a controversial alternative to abstinence as an appropriate treatment goal for alcoholics. In this study we reexamine the evidence underlying a widely cited report by Sobell and Sobell of successful controlled drinking by a substantial proportion of* gamma *(physically dependent) alcoholic subjects in a behavior therapy experiment. A review of the evidence, including official records and new interviews, reveals that most subjects trained to do controlled drinking failed from the outset to drink safely. The majority were rehospitalized for alcoholism treatment within a year after their discharge from the research project. A 10-year follow-up (extended through 1981) of the original 20 experimental subjects shows that only one, who apparently had not experienced physical withdrawal symptoms, maintained a pattern of controlled drinking; eight continued to drink excessively—regularly or intermittently—despite repeated damaging consequences; six abandoned their efforts to engage in controlled drinking and became abstinent; four died from alcohol-related causes; and one, certified about a year after discharge from the research project as gravely disabled because of drinking, was missing.*

Conventional wisdom in the health professions has long held that persons who have become physically dependent on alcohol must be advised to abstain completely. In 1962, Davies sparked debate by reporting that 7 of 93 alcoholic patients were found on long-term follow-up to be able to drink moderately (*1*). Since then, the controversy has been intensified by conclusions of other investigators that some alcoholics can safely resume social, moderate, or controlled drinking as an alternative to abstinence (*2, 3*).


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