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THERAPEUTIC EFFICACY OF ASPARAGINASE ENCAPSULATED IN INTACT ERYTHROCYTES

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Abstract—Asparaginase has been encapsulated in intact erythrocytes by a gentle loading technique. The loaded cells were found to survive removal by the recticuloendothelial system when returned to the circulation of mice. In addition the enzyme removed all detectable asparagine from the plasma *in vivo* for at least two weeks after the injection of the loaded cells. *In vitro* evidence suggested that the asparagine entered the cell and was metabolised by the loaded enzyme *in situ*. No evidence was found to suggest that the enzyme left the cell. When the encapsulated asparaginase was tested against the 6C3HED tumour in C3H mice the encapsulated preparation was superior to the free enzyme in treating the tumour and was the only treatment to produce 'cured' mice. Encapsulated asparaginase also lowered glutamine levels both *in vivo* and *in vitro*. The possibility that LDH virus was responsible for the excellent results obtained with the encapsulated enzyme was investigated and eliminated.

The therapy of neoplasia with L-asparaginase is associated with a number of problems, such as occasional severe organ toxicity which in some cases can lead to death. The toxic effects of the enzyme normally derived from either *Escherichia coli* or *Erwinia carotovora* are functions of dose and duration of therapy. The therapeutic action of asparaginase depends on its plasma half-life which is relatively short for the free enzyme. A single injection of the *E. coli* enzyme in patients gives a plasma half-life of 8–30 hr independent of dose, disease, sex of the patient, hepatic or renal function [1, 2]. As with most drugs and therapy demands a compromise between the clinical efficacy and the containment of toxic effects between reasonable limits. Even so asparaginase has to be administered at least 2–3 times weekly, over several weeks. Attempts have been made to reduce the toxicity of asparaginase by immobilising it within a non-antigenic matrix prior to injection. Methods employed include the entrapment of the enzyme in liposomes [3] or erythrocyte ghosts [4]. The liposomes were rapidly removed by the recticuloendothelial system [3] but the erythrocyte ghosts (monkey) loaded with asparaginase were found to have a half-life span of 7 days. The half-life span of control cells (empty ghosts) was 9 days and normal monkey crythrocyte has a half-life span of 21–22 days [4]. Microencapsulated material does not survive early removal by the recticuloendothelial system and where synthetic materials have been used problems have arisen because the microcapsules are too large to enter the microcirculation. Other techniques employing haemodialysis and immobilised enzyme [5] or depot asparaginase embedded in polyhydroxylethyl methacrylate gels [6] have shown only limited success. Erythrocytes were used to encapsulate enzymes as a means to correcting certain inborn errors of metabolism [7]. Early encapsulation techniques with erythrocytes were often rigorous resulting in small amounts of material being encapsulated and poor survival times for the cells when returned to the circulation. Less rigorous techniques have evolved which have increased both the amount of drug loaded in the cells and also the survival times of the cells *in vivo*. These techniques have recently been reviewed [8] but successful techniques employ preswelling of the cells without lysis followed by a final loading stage [9] or by dialysing cells against hypotonic drug solutions [10]. Other gentle loading methods employ electrical techniques [11]. In this laboratory [12] we used a method based on the preswelling technique. We mixed erythrocytes with hypotonic Hanks balanced salt solution but we reversed the Na^+ and K^+ concentrations (Na^+/K^+ reversed—HBSS)$^+$. We recovered the swollen cells by centrifugation and protected them by layering a portion of haemolysed erythrocytes on top of the cells. This layer provided cytoplasmic constituents which would replace any losses due to the encapsulation procedure and also provide a physical barrier between the cells and the aqueous solution of the drug to be encapsulated. The encapsulation step consisting of layering portions of the drug solution on top of the haemolysate and gently mixing the drug with the cells. On centrifugation the cells sedimented leaving a barrier of supernatant to protect the cells on the addition of a further portion of drug. Drug. in aqueous solution was added in successive portions until the point of lysis of the cells was reached [12] when they were resealed by the addition of the calculated amount of HBSS. Our cells were found to have excellent survival times *in vivo* in the four animal species tested. In this communication we have used our encapsulation technique to encapsulate L-asparaginase in erythrocytes and we have used the loaded cells to treat the 6C3HED lymphoma tumour in C3H mice.

MATERIALS AND METHODS

Encapsulation of asparaginase in intact erythrocytes. A stock of *Erwinia carotovora* L-asparaginase

was iodinated [13] using chloroamine T and ^{125}NaI and the radiolabelled enzyme was mixed with "cold" enzyme as a tracer. Solutions of 2000 units per ml of asparaginase in water (1 unit of asparaginase is the amount of enzyme required to hydrolyse 1 μmole of substrate per min at 37°) were prepared and for *in vivo* experiments sterilized by ultrafiltration (Millipore S.A., GS type, pore size 0.22 μm). Erythrocytes were obtained by centrifuging blood obtained by cardiac puncture from C3H female mice or, when other species were used, from pooled blood from that species. The encapsulation procedure has been described [12] but consisted in swelling 1 vol of packed erythrocytes (haematocrit 76) with 4 vol of 0.66 isotonic Na^+/K^+-reversed HBSS. The swollen cells were recovered by centrifuging (500 *g* for 5 min) and protected by a layer of haemolysed erythrocytes (1:1 v/v with water). For each ml of packed cells 100 μl of haemolysate was used. The asparaginase solution was layered on top of the haemolysate (200 μl of solution for each ml of packed cells) and then gently mixed with the haemolysate and cells. The suspension was centrifuged at 500 *g* for 4 min and a second portion of asparaginase solution was layered on top of the supernatant. The mixing and centrifugation steps were repeated. Asparaginase was added in successive portions until the point of lysis was reached. This point was observed when on centrifugation some white ghosts were observed on top of the red cells after centrifugation. At this point the cells were sealed by the addition of the calculated amount of 10 times hypertonic HBSS. The resealed cells were washed twice with HBSS and recovered by centrifuging. The amount of radioactivity associated with the cells was determined in a Gamma particle counter (Tracerlab Instruments Gamma set 500). As controls non-swollen cells were suspended in the asparaginase solution for the same period of time used in the encapsulation. The encapsulation procedure normally took 1 hr.

Bioassay. Although the use of ^{125}I informed us of the amount of enzyme encapsulated it was necessary as a potential therapeutic procedure to determine the biological activity of the encapsulated enzyme. Non-radiolabelled enzyme was used for the encapsulation and the loaded cells were lysed by freezing and thawing in 0.1 M sodium tetraborate buffer (pH 8.5). The biological activity was determined by adding [^{14}C]glutamine solution (0.04 M, 1 μCi/μmole) to the cell lysate and incubating for 30 min at 37° in a shaking waterbath. The amount of [^{14}C] glutamine and [^{14}C]glutamate was determined by separating the compounds by cellulose acetate electrophoresis using a pyridine, acetic acid, water mixture (25:1:225 v/v) (pH 6.0) and scanning the dried strips with a II windowless gas flow radiochromatogram scanner (Tracerlab Instruments).

Survival of loaded cells in CH3 female mice. For *in vivo* experiments non-radioactive enzyme was encapsulated under aseptic conditions. To trace the cells and to distinguish them from normal cells we incubated the resealed cells in HBSS saturated with fluorescein isothiocyanate (FTIC) [12] using 1 ml of packed cells to 0.6 ml solution over a 1 h period at room temperature (20°). After washing the cells with HBSS to remove excess FITC, portions of the labelled cells (0.2 ml packed cells containing 50 units of asparaginase) were injected into a tail vein of mice. Blood samples were taken from the tip of the tail at periods from 2 min after the injection up to 30 days after the injection. Each sample was counted in a haemocytometer under both uv and tungsten light using a Zeiss microscope with uv attachment. The experiment was repeated using Balb C female mice (50 units asparaginase each) and male Wistar strain rats (200 units asparaginase each) injected into the femoral vein.

Uptake of amino acids by asparaginase loaded erythrocytes. In this experiment erythrocytes loaded with asparaginase (40 units) were suspended in HBSS containing ^{14}C-asparagine (0.2 ml packed cells to 1 ml HBSS or plasma). The suspension was incubated at 37° in a shaking waterbath for various periods. At the end of these periods the cells were recovered by centrifugation and washed in HBSS. The cells were lysed by freezing and thawing in 0.2 ml water. The suspension was centrifuged and the supernatant examined for asparagine and aspartate. The asparagine and aspartic acid were separated by cellulose acetate electrophoresis and the radioactivity of the amino acids determined by scanning the dried strip on a II windowless gas flow radiochromatogram scanner. A similar separation was carried out on the cell supernatant.

The depletion of plasma asparagine and glutamine in vivo. Female C3H mice were injected with 50 units of asparaginase encapsulated in erythrocytes, or with 50 units of free enzyme dissolved in 0.2 ml saline, or with cells that had been subjected to the encapsulation procedure but where asparaginase had been omitted (sham encapsulation). Mice in groups of 9 were sacrificed at various times during the experiment and their plasma analysed for amino acids by a Locarte Automatic amino acid analyser (University of Birmingham, Macromolecular Analysis Service). In other experiments the mice were bled by cardiac puncture after various periods and the asparaginase activity determined in the erythrocytes using [^{14}C]-labelled asparagine as substrate as previously described.

Therapeutic activity of encapsulated asparaginase against the 6C3HED lymphoma tumour in mice. Lymphoma 6C3HED cells were injected i.p. (2×10^6 cells per mouse) and the cells were passaged to fresh mice at eight day intervals. These cells were suspended in sterile saline and 4×10^5 cells in 0.2 ml saline were injected i.p. into mice which were then divided into experimental groups of 8. The mice were then injected in the tail vein either on the same day, or 8 days later with either 0.2 ml of saline, 0.2 ml of sham encapsulated cells suspended in saline, 0.2 ml of saline with 25 units of asparaginase in solution, or 25 units of asparaginase encapsulated in erythrocytes. All mice were originally 25–28 g in weight. The mice were allowed food and water *ad libitum* and their survival times recorded. Mice that survived for 60 days were scored as a "cure". In a further experiment the mice were bled by cardiac puncture at different intervals after the injection of tumour cells and the lactate dehydrogenase activity determined in the serum by the Sigma LDH kit (Sigma Chemical Co., Poole, U.K.).

RESULTS

The amount and activity of asparaginase encapsulated in erythrocytes.

Radioactivity determinations

After correcting for radioactivity associated with non-swollen erythrocytes allowed contact with asparaginase in HBSS over the encapsulation period, it was calculated that 1 ml of packed cells (haematocrit value 76) had encapsulated 200 units of enzyme. This represents 10% w/w of the enzyme added to the cells. Resealed cells were treated with 0.5% w/v trypsin in HBSS for 30 min at 37° but this treatment failed to release radioactivity into solution suggesting that the asparaginase was inside the cells. In later experiments using solutions of 2500 units of enzyme we increased the loading to 250 units of enzyme per ml of packed cells.

Bioassay

After corrections for the small amount of endogenous enzyme activity in normal erythrocytes and an adjustment of the haematocrit value to 76 it was found that 1 ml of packed cells encapsulated 180 units of enzyme which is in good agreement with the radiochemical assay result.

Survival of loaded cells in mice and rats

The results in Fig. 1 showed that the loaded cells had excellent survival characteristics in the two species of mouse used and in the Wistar rat. The cell survival half-life span ($t_{1/2}$) values were Wistar rats 9–10 days, Balb C mice 23–24 days and C3H mice 14 days.

Uptake of asparagine by loaded erythrocytes

The uptake of asparagine by 0.2 ml of packed erythrocytes loaded with asparaginase is shown in Fig. 2. The results show that once the cells had taken up asparagine it was rapidly deaminated to aspartic acid. After 30 min about 80% of the radioactivity was associated with aspartic acid and this had increased to 95% by 1 hr. In the supernatant no radiolabelled aspartic acid was detected. There was no leakage of radiolabelled aspartate or asparaginase from the cell. Plasma had no significant (Fig. 2) effect on rate of asparagine transport when compared with HBSS as the suspending medium. It also appears unlikely that very little enzyme was associated with the outer membrane of the erythrocytes. It is well known that the higher the external Na^+ concentration the greater the capacity of the amino acid transport system to transport amino acids into the cell. There conditions would favour the entry of asparagine into the cell. Aspartate was produced in a K^+ enriched environment and was unlikely to be transported by the same mechanism as asparagine. The most likely transport mechanism for aspartate is "facilitated diffusion" and the polar nature of aspartate may well make the passage of this acid across the lipophilic erythrocyte membrane very slow.

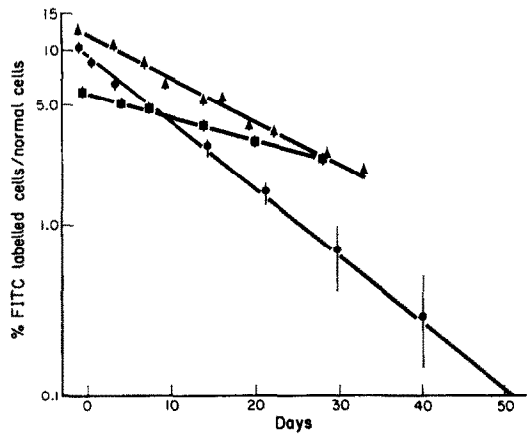

Fig. 1. The semi-log plots shown from Wistar strain male rats, C3H female mice and Balb C female mice are the survival plots of erythrocytes containing encapsulated L-asparaginase. The survival half-life span ($t_{1/2}$) values were Wistar rats $t_{1/2}$ = 9–10 days: C3H mice, $t_{1/2}$ = 14 days and Balb mice $t_{1/2}$ = 23–24 days. The initial (day 0) and later values represent the % of FITC-labelled cells present in the total blood volume of the animals. Eight animals were used in each experiment ● rats, ■ Balb C, ▲ C3H mice.

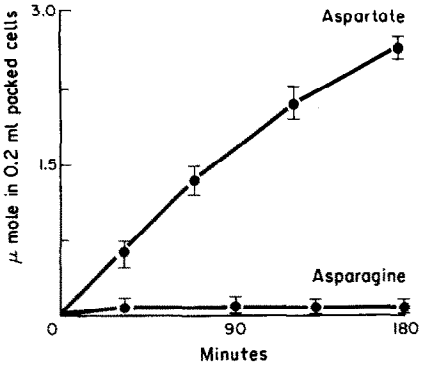

Fig. 2. Uptake of asparagine and its conversion to aspartate by erythrocytes loaded with asparaginase. The results shown are for cells suspended in HBSS. When plasma was substituted for HBSS the corresponding results were 0.75 μmoles (30 min); 1.31 μmoles (60 min); 1.99 μmoles (120 min) and 2.90 μmoles aspartate (180 min) (P = n.s. for all values, Student *t* test).

Plasma levels of asparagine and glutamine in vivo

The plasma levels of asparagine after single injection of 50 units of enzyme, either free or encapsulated, in erythrocytes is shown in Fig. 3. Aspartate, glutamine and glutamate values are also plotted. Where the enzyme was encapsulated both asparagine and glutamine were not detected in the first sample of plasma taken 2 hr after the administration of the enzyme. Although the glutamine levels subsequently recovered although remaining below control values for 14 days no asparagine could be detected in plasma over this period. The fall in glutamine levels was probably due to some glutamine deaminase activity by the encapsulated enzyme, as the glutamate levels rose to values in excess of control values. Aspartate values were elevated immediately after the administration of free enzyme but returned to normal values after 72 hr. Aspartate levels did not rise after the administration of encapsulated enzyme. As

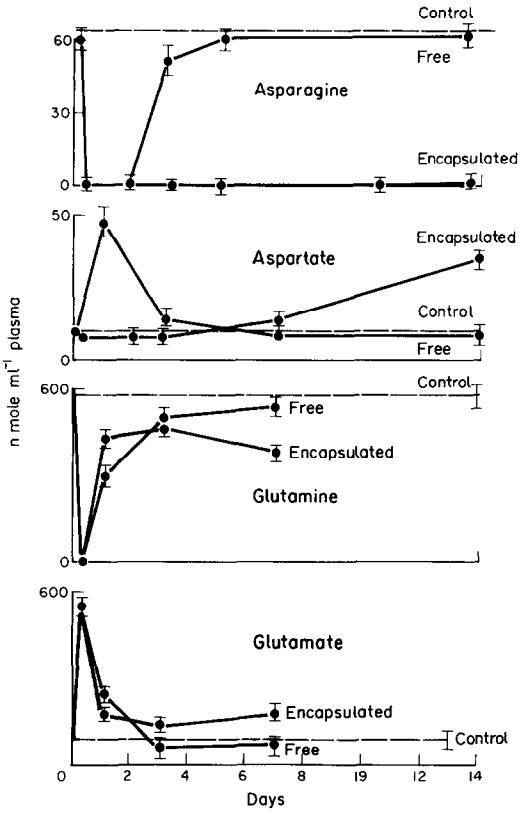

Fig. 3. Plasma levels of asparagine, aspartate, glutamine and glutamate in mice after the administration of either the free asparaginase or asparaginase encapsulated in erythrocytes via tail vein.

shown in the *in vitro* experiments aspartate was probably initially incorporated into the amino acid pool within the erythrocyte to be released later when the cells were broken down. Since both glutamate and aspartate are essential biological intermediates in a variety of reactions and their levels controlled by transamination reactions it is likely that deamination of asparagine or glutamine may have minor roles in controlling the levels of these intermediates. The key result in this experiment was the complete removal of asparagine from the plasma since the 6C3HED tumour is sensitive to plasma levels of asparagine. The variation of plasma levels of asparagine shows that free enzyme treatment depressed asparagine levels only to 72 hr for at this time the amino acid plasma levels were close to normal values. Sham encapsulated cells did not deplete plasma amino acids.

In experiments where the residual asparaginase activity was determined in erythrocytes at various periods after the initial injection it was found that after 7 days cells retained 80% of their original activity (40 units) and after 14 days 45% (27.5 units). These values correspond closely to the *in vivo* cell survival curves (Fig. 1) and the plasma $t_{1/2}$ value of the enzymes is identical with the $t_{1/2}$ values for the encapsulating cells.

Therapeutic action of asparaginase encapsulated in erythrocytes against the 6C3HED lymphoma tumour

The groups of mice treated with saline, sham encapsulated cells and untreated died in 18 ± 2.5, 18.3 ± 2.0 and 18.9 ± 2.5 days respectively. The mean survival time for the mice treated with free enzyme was 29.4 ± 5.7 days. All these treatments were commenced on the same day as the tumour cells were injected. In the group treated with encapsulated enzyme 5 mice out of 8 survived for 60 days and were classified as "cured". Values quoted are mean values ± S.E.M. The encapsulated enzyme treated group was the best treatment ($P < 0.025$ when compared with all other groups) (Fischer exact probability test.) In the groups where there were no survivers the free asparaginase treatment prolonged life ($P < 0.001$ Student t test) compared with groups that were not treated with asparaginase. The LDH values for plasma are given in Table 1. In Table 1 the mice were injected with 6C3HED lymphoma cells and the LDH plasma values determined as the tumour developed. The LDH values increased as the tumour developed showing an association between the LDH virus and the tumour. In another experiment (Table 2) the tumour cells were injected into the mice and treatment with free and encapsulated enzyme was commenced 8 days later after the tumour had developed. The results of this experiment (Table 2) show that the encapsulated enzyme was still superior to the free enzyme in prolonging life. The LDH virus itself may have had an anti-tumour effect by inhibiting clearance of the free enzyme from the circulation [14] but it seems unlikely that clearance of the encapsulated enzyme was accelerated. This possibility was not investigated. However, there was no significant difference between the early and late treatments with encapsulated enzyme (Table 2). (Mann–Whitney U test). In an attempt to avoid possible antitumour effects by the virus on the native enzyme, we commenced treatment on the same day as the tumour cells were injected.

Table 1. Serum LDH elevation following inoculation of 6C3HED tumour cells into C3H mice

Days after inoculation	LDH (units/l of serum)	Degree of elevation
0 (control)	217.8 ± 8.7	
1	365.0 ± 15.9	1.7 times
4	1364.4 ± 104.5	6.3 times
75	1121.3 ± 89.4	5.2 times

Table 2. Effect of encapsulation of asparaginase on the treatment of lymphoma tumour (6C3HED) in the C3H mouse

Treatment	% of increase in life span	
	Free enzyme	Encapsulated enzyme
Early	55 ± 2.9	>294*
Late	>121†	all cured

* 5 mice out of 8 survived longer than 60 days.
† 2 mice out of 5 survived longer than 60 days.
4×10^5 6C3HED cells per mouse were implanted i.p. into C3H female mice and treated by i.v. on the same day (early) treatment or eight days later (late treatment). Each group contained eight mice for the early therapy and five mice for the late therapy.

DISCUSSION

The results have shown that asparaginase has been encapsulated in erythrocytes at therapeutically useful levels. The encapsulated preparation has been shown to be superior to the free enzyme in reducing asparagine plasma levels *in vivo* and in treating the 6C3HED lymphoma tumour in mice. The biochemical mechanisms for the therapeutic properties of the preparation appears to be that plasma asparagine enters the cells in which the enzyme is entrapped and is deaminated to aspartic acid. Plasma asparagine levels fall to very low levels which are maintained by the prolonged activity of the circulating enzyme. The $t_{1/2}$ value of the encapsulated enzyme was identical to the $t_{1/2}$ value of the encapsulating cells. We used a heterogenous cell population in the encapsulation procedure but an improvement in our technique would be to separate young cells from older cells and to use these for the encapsulation procedure since they would prolong considerably the survival time of the preparation *in vivo*. The separation of erythrocytes according to their age has been achieved previously [15, 16] and we have previously shown that young cells load drugs in preferences to older less elastic cells [12].

Although we used animal cells in our experiments we were able to achieve similar loading values with human cells but we have yet to test this preparation therapeutically. Since the life-span of asparaginase in erythrocytes is much greater than the free enzyme the frequency of dosing is much less with encapsulated enzyme than free enzyme.

Attempts have been made to treat pathological conditions with materials encapsulated in erythrocytes. In preliminary experiments glucocerebrosidase was entrapped in erythrocytes and used to treat patients [17]. The results of these experiments were inconclusive. In other work desferrioxamine encapsulated in erythrocytes was used to treat iron toxicity [18] with some success and galactosidase encapsulated in erythrocytes was successfully used to treat an infant with elevated levels of galactose due to an inborn deficiency of the enzyme [19]. A common feature of the clinical experiments that the use of erythrocytes produced no toxic effects and the author in one paper commented favourably on the safety of the technique [16].

A further possible advantage of a therapy using asparaginase entrapped in erythrocytes is that since the biochemical mode of action is different to that of other agents, then cytotoxic therapy would be theoretically possible at the same time.

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REFERENCES

1. M. K. Schwartz, E. D. Lash, H. F. Oettgen and F. A. Tamo, *Cancer* **25**, 244 (1970).
2. J. C. Wriston, Jr. and T. O. Yelin, *Adv. Enzymol.* **39**, 185 (1973).
3. E. D. Neerunjun and G. Gregoridadis, *Biochem. Soc. Trans.* **21**, 133 (1976).
4. S. J. Updike, R. T. Wakamiza and E. N. Lightfoot, Jr., *Science* **193**, 681 (1976).
5. D. Sampson, L. S. Hersch, D. Cooney and G. P. Murphy, *Trans. Am. Soc. Artif. Intern. Organs* **18**, 54 (1972).
6. K. F. O'Driscoll, R. A. Korus, T. Ohnuma and I. M. Walczack, *J. Pharmac. exp. Ther.* **195**, 382 (1975).
7. G. M. Ihler, R. H. Glew and F. W. Schnure, *Proc. natn. Acad. Sci. U.S.A.* **70**, 2613 (1973).
8. G. M. Ihler, *Pharmac. Ther.* **20**, 151 (1983).
9. M. C. Rechsteiner, *Expl. Cell. Res.* **83**, 487 (1975).
10. J. R. DeLoach and G. M. Ihler, *Biochim. biophys. Acta.* **496**, 136 (1977).
11. K. Kinosita and T. W. Tsong, *Nature* **272**, 258 (1978).
12. E. Pitt, C. M. Johnson, D. A. Lewis, D. A. Jenner and R. E. Offord, *Biochem. Pharmac.* **32**, 3359 (1983).
13. W. M. Hunter and F. C. Greenwood, *Nature, Lond.* **194**, 495 (1962).
14. V. Riley, *Nature* **220**, 1245 (1968).
15. S. Piomelli, L. M. Corash, D. D. Davenport, S. Miraglia and E. L. Amorosi, *J. clin. Invest.* **47** 940 (1968).
16. D. A. Galbraith and D. C. Watts, *Biochem. J.* **191**, 63 (1980).
17. E. Beutler, G. L. Dale, G. Guinto and W. Kuhl, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4620 (1977).
18. R. Green, J. Lamon and D. Curran, *Lancet* **2**, 327 (1980).
19. R. C. Harris, *New Engl. J. Med.* **296**, 942 (1977).