In vitro Cytocidal Effect of L-Glutaminase on Leukaemic Lymphocytes

MOUSE lymphomas, such as 6C3HED, undergo complete regression after one or more injections of L-asparaginase[1], and remissions have been reported in some patients with acute leukaemia after treatment with L-asparaginase[2-4]. Roberts *et al.*[5] found that purified bacterial L-glutaminase inhibited the growth of Ehrlich mouse carcinoma. Before considering this enzyme for therapeutic studies in man, it is necessary to study *in vitro* effects of the enzyme on human blood cells. Previous work[6] demonstrated that the cells of 6C3HED lymphoma do not survive incubation with very low concentrations of L-asparaginase (1.7 mIU/ml.), while the cells of the *in vivo* resistant variant of 6C3HED were not killed by 170 mIU of enzyme/ml. The blood lymphocytes from patients with chronic lymphocytic leukaemia were found to be more sensitive than normal lymphocytes to incubation with 170 mIU of L-asparaginase/ml. but not as sensitive as 6C3HED mouse lymphoma which responds *in vivo* to the enzyme. In this study, the slide chamber method[7] was used to compare the sensitivity of normal and leukaemic lymphocytes to glutaminase.

The blood cells tested were obtained from five persons with normal haemograms, from eight patients with chronic lymphocytic leukaemia, and from three patients with acute leukaemia. Suspensions of the blood cells plus varying amounts of L-glutaminase were incubated at 37° C for 7 days. The suspensions were then examined in a special slide chamber and the surviving cells were counted. The criteria of viability depended on the morphological integrity of the cells under phase microscopy[7].

The L-glutaminase was derived from bacteria and was described as preparation GA : 1.2 in a previous report[5]. The preparation also had L-asparaginase activity equal to 80% of the glutaminase activity. In all experiments, control tests were done with equivalent amounts of purified L-asparaginase to evaluate toxic reactions due to L-asparaginase.

A summary of the findings is given in Table 1. Most of the normal lymphocytes survived incubation with 1,700 mIU of glutaminase/ml. The cells of one patient (M.U.) with chronic lymphocytic leukaemia had approximately the same resistance to the enzyme as normal lymphocytes. In the case of the other seven patients with chronic lymphocytic leukaemia and of three patients with acute leukaemia, very few cells survived incubation with 5 to 0.5 mIU of enzyme/ml.

Table 1 Percentage of Lymphocytes or Blasts that survived Incubation at 37° C for 7 Days with Varying Amounts of L-Glutaminase

Source of cells	Cells surviving (%) L-glutaminase (mIU/ml.) 0	1,700	170	17	5	1.7	0.5	0.17
Normal persons (5)	53	33	35					
Patients with chronic lymphocytic leukaemia								
M. U.	32	18	11					
P. K.	46	—	3	3	3	13	33	59
K. O.	48	—	—	2	1	2	1	38
S. T.	39	—	—	0	0	1	5	29
C. S.	43	—	0	1	0	2	—	—
K. R.	76	—	0	4	1	3	—	—
H. U.	42	—	0	0	0	0	—	—
L. A.	65	—	4	8	11	7	43	66
Patients with acute leukaemia *								
P. H.	55	—	0	—	0	1	—	—
T. K.†	45	—	3	12	35	34	—	—
W. M.†	27	—	2	2	1	2	10	—

* P. H. had acute plasma cell leukaemia; T. K. had acute myelomonocytic leukaemia; W. M. had acute lymphoblastic leukaemia.
† Cells were incubated for 5 days.

The media of the incubated cells were analysed to determine the effect of the enzyme on the concentration of glutamine, ammonia and glutamate. As would be expected, the enzyme caused a decrease in glutamine (to < 0.02 mmol) in suspension of both the leukaemic and normal lymphocytes. The ammonia and glutamate increased correspondingly to 1.0 and 0.8 mmol, respectively. In further experiments, the addition of 1 mmol of NH_4Cl or 0.8 mmol of sodium glutamate or both had no effect on the survival of leukaemic lymphocytes. These findings suggest that the decrease of glutamine in the media killed leukaemic but not normal lymphocytes *in vitro*.

According to our findings, the leukaemic lymphocytes from seven of eight patients were killed directly or indirectly by L-glutaminase, usually at the level of 1.7 mIU/ml. It is noteworthy that this dose of L-asparaginase was cytocidal to cells of 6C3HED mouse lymphoma[6] which undergoes remission *in vivo* with L-asparaginase therapy.

Normal lymphocytes resisted the concentration of 1,700 mIU of L-glutaminase/ml. although leukaemic lymphocytes usually responded to 1.7 mIU/ml. Previous work from this laboratory[8] showed that the leukaemic lymphocytes are also more sensitive *in vitro* than normal cells to other reagents including L-asparaginase, prednisolone, dimethyl sulphoxide and heat. For example, leukaemic lymphocytes were sensitive to prednisolone at the level of 1 μg/ml. and to L-asparaginase at 170 mIU/ml. Glutaminase, however, acted at a much lower concentration than the other reagents and showed a much greater difference in its effect on normal and leukaemic cells.

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Inequality of Inspired and Expired Gaseous Nitrogen in Man

IN studies of animal respiration, it is conventional to assume that the amounts of nitrogen inspired and expired are equal, and this assumption has passed from one textbook to another[1-12]. The argument is that there is no known metabolic reaction of the human body involving molecular nitrogen, and

that in ordinary circumstances nitrogen is merely a diluent of the oxygen in the air breathed[13]. Three recent studies[14–16] cast doubt on this assumption and have prompted us to use a direct system to detect changes in the amounts of inspired and expired nitrogen gas.

This investigation revealed an obvious but little known biphasic history of the theory of nitrogen involvement. The initial hypothesis, based largely on the works of Lavoisier[17–20], was that nitrogen gas was neither produced nor absorbed. There followed a period in which the consensus supported a change in the amount of nitrogen, although it was debated whether this change was positive or negative—production or absorption. Current thought has reverted to Lavoisier's original dictate that the amount of nitrogen expired is equal to the amount inspired. In neither of these sources, however, are direct experimental data presented, nor are earlier sources cited where such data are available.

Allen and Pepys determined[21,22] that: (*a*) no change occurs in atmospheric air in respiration except the substitution of carbonic acid for an equal amount of oxygen; but (*b*) when pure oxygen is respired a portion of it is replaced by an equal amount of azote (N_2). In small animals, Edwards[12,23,24] found that whether nitrogen was exhaled or absorbed depended on the season of the year (temperature) and the age of the animal.

In 1839 Boussingault[25,26] measured the nitrogen in the food of a milch cow, a horse and a pigeon, and the nitrogen excreted by these animals. The output was less than the intake: 27 g/day for the cow, 24 g/day for the horse, and 1.14 g/day for the pigeon. He concluded that gaseous nitrogen is not assimilated in the process of respiration. Regnault and Reiset (1849)[27] studied small mammals, birds, amphibians and worms. Warm blooded animals exhaled nitrogen in from 1/100 to 1/50 the proportion of oxygen consumed. Animals in a state of inanition and occasionally cold blooded animals were found to absorb nitrogen. Barral[27,28] used the same indirect method as Boussingault. In five humans he found the ingested nitrogen to be greater than the nitrogen in urine and faeces. The excess was said to be passed off by the lungs and skin. Berthollet *et al.*[29], Dulong[27], Berthollet and Nysten[23], Jurine and Nysten, Nysten and Treviranus[29] also observed an exhalation of azote (nitrogen).

This evidence notwithstanding, the exhalation of nitrogen was far from definite. Priestley[23] supposed that the gas was absorbed, and he was supported by Davy, Cervier, Henderson, Pfaff, Spallanzani, Humboldt and Provencal[23], and Marchand[10,29].

After this period of lively research there was a marked lack of interest in the subject, continuing until the current idea of non-change came to the fore. The link that seems to be missing is experimental evidence to support this shift and lack of interest. If this is true, then to attribute some of the experimental evidence for absorption or exhalation to error[30] or accident[23,31], while accepting Lavoisier's verdict without direct confirmation, appears to be a questionable solution to the problem.

Criticisms of Lavoisier's viewpoint and the validity of the classical technique for the determination of nitrogen balance (after Lusk)[26], have been made by Costa, Ullrich, Kantor and Holland[15]. The criticisms are based on evidence substantiating nitrogen loss in sweat and positive nitrogen balances in various mammals that failed to demonstrate a commensurate body weight gain in proportion with the nitrogen retention.

Several reports[14,15,32] reveal interesting inconsistencies with the historic or classic concept of nitrogen non-involvement in respiratory gas exchange, and suggest that molecular nitrogen is an unrecognized metabolite and that quantities of nitrogen sufficient to balance the nitrogen equation could conceivably escape undetected among the expired respiratory gases. Costa *et al.* showed that some of the nitrogen fed is eliminated through this neglected pathway[14,15]. With a closed system technique in which a nitrogen-free atmosphere was made with helium–oxygen washout, a substantial increase in the concentration of nitrogen was measured with a mass spectrometer. The absolute amounts of elemental nitrogen produced in mice were approximately 14–28 mg/kg/24 h. These quantities are sufficient (5–10% of intake) to balance the nitrogen equation. In humans the estimated total production of nitrogen was in the range 8–24 mg/kg/24 h.

Levitt[33] used a constant perfusion technique and reported that hydrogen occurs in the expired air of humans. Of all the hydrogen produced in the gastrointestinal tract, about 14% appears in the expired air[33]. Mitchell[34] concedes that nitrogen gas excretion via the lungs may occur in certain herbivores due to nitrogen liberating bacteria. Robin *et al.*[35] have found ammonia in the expired air of dogs. In non-steady state conditions, as when the barometric pressure changes, or when the temperature of the body fluctuates, gaseous N_2 may be dissolved or released from solutions in the body, and so appear as exhaled or retained nitrogen.

With this confusing background in mind we investigated whether or not gaseous nitrogen was being expired in excess of that inspired in humans, both at rest and during moderate exercise, always in steady state conditions.

All samples were collected by a syringe-metallized bag technique as described by Johnson *et al.*[36]. Gas analysis was done with a Beckman paramagnetic oxygen analyser (model C2, sensitivity 0.15 mm Hg partial pressure of oxygen, O coefficient of variation) and a Godart NV carbon dioxide thermal analyser (model A2, gain 0.2, sensitivity 0.07 mm Hg partial pressure of CO_2, 1% coefficient of variation). N_2 was calculated by difference:

$$F_{N_2} = 1 - F_{O_2} - F_{CO_2} \quad (1)$$

The other calculations were made from Haldane's equations[8].

At rest the subject was seated between two Tissot gasometers. Through a standard double Douglas valve he inspired from one gasometer and expired into the other. The room was kept at 24° C, 40% relative humidity. The system was calibrated by forcing random volumes of air from the inspired Tissot to the expired and analysing samples of each. During the treadmill walk the inspired air flowed from a Tissot gasometer of 800 l. capacity through a sampling chamber and then a Parkinson–Cowan dry gasometer and finally a sampling chamber. The subject breathed through a low resistance-high velocity Otis–McKerrow valve. Room temperature was controlled at 15.5° C, with a 50% relative humidity. The system was calibrated by forcing air through at rates like those of a man walking. Samples were collected and analysed as described here.

The results of thirty-six resting experiments corrected for calibration differences (Table 1) showed no average nitrogen production, but rather a mean negative figure of −27 ml./min which would indicate absorption. There were nine positive, twenty-seven negative and no equal results. This was significant to the 0.01 level by a χ^2 analysis. The historical literature survey had also demonstrated production or absorption under different conditions, so it was then our supposition that if any nitrogen had been produced it might be accentuated in walking conditions and thus be consistently measurable with our equipment. This proved to be the case. In the thirty-five walking experiments thirty gave positive results, that is, expired nitrogen was greater than inspired. On the basis of χ^2 analysis this result was significant at the 0.001 level. The mean difference was 132 ml./min at a pulmonary ventilation of about 16 l./min. The mean N_2 excess was about 2 ml./kg min (Fig. 1).

Our results are similar to those of Costa *et al.*[14,15], though our values are rather higher. Our experiments also show wide inter and intra-individual variations, for which we have no explanation. The demonstration of molecular hydrogen in man[33], with 14% of the total hydrogen production of the intestines excreted by the lungs, suggests that some mechanism may exist for diffusing nitrogen. In any case, there do seem

to be grounds for questioning the doctrine of equality for inspired and expired nitrogen.

One important practical question raised by this apparent production of nitrogen in man is, What effect does it have on respiratory calculations? How much difference does it make? We have taken one of the most extreme cases for resting and one for walking and made a comparison between the actual measured values and values obtained by the Haldane calculations (Table 2). In our conditions the carbon dioxide produced was closely approximated either by actual measurement or by Haldane's assumption. In these two extreme cases, however, the correct oxygen consumed in fact was as much as 12% lower than that calculated from the expired air alone by Haldane's method.

Table 1 Inspired and Expired Nitrogen in Humans Sitting Resting

A Subject and test *	*B* Expired N_2 l./min †	*C* Inspired N_2 l./min †	*D* (*B*−*C*) l./min
S. P. 1	5.715	5.781	−0.066
	4.046	4.033	+0.013
	3.734	3.533	+0.201
L. D. 1	6.128	6.156	−0.028
	6.015	6.012	+0.003
	6.025	6.101	−0.076
H. I. 1	5.723	5.830	−0.107
	5.672	5.756	−0.084
	6.201	6.210	−0.009
J. P. 1	4.004	3.959	+0.045
	3.239	3.232	+0.007
	3.190	3.251	−0.061
S. P. 2	4.436	4.414	+0.022
	4.540	4.562	−0.022
	4.727	4.726	+0.001
L. D. 2	5.663	5.704	−0.041
	5.347	5.432	−0.085
	5.005	5.052	−0.047
H. I. 2	5.929	5.938	−0.009
	5.455	5.566	−0.111
	5.549	5.604	−0.055
J. P. 2	3.762	3.855	−0.093
	3.665	3.716	−0.051
	3.528	3.617	−0.089
S. P. 3	4.727	4.409	+0.318
	4.561	4.593	−0.032
	4.025	4.048	−0.023
L. D. 3	5.902	5.935	−0.033
	5.684	5.800	−0.116
	6.134	6.280	−0.146
H. I. 3	5.575	5.658	−0.083
	5.868	5.915	−0.047
	5.953	5.959	−0.006
J. P. 3	4.616	4.678	−0.062
	4.727	4.779	−0.052
	4.541	4.502	+0.041
N	36	36	36
Mean	4.989	5.016	−0.027

* Tests were about 1 week apart for any given subject.
† Corrected for differences in calibration between intake and output Tissot gasometers.

Table 2 Actual and Calculated Values for Oxygen consumed and Carbon Dioxide produced when Expired Nitrogen is greater than Inspired Nitrogen

A Item measured or calculated (l.)	*B* Resting (J. P.) (*a*) Actual value *	(*b*) Haldane calculation	*C* Walking (L. D.) (*a*) Actual value *	(*b*) Haldane calculation
(*a*) Air inspired	5.573		19.312	
(*b*) Air expired	5.673	5.673	19.342	19.342
(*c*) N_2 inspired	4.404	(4.534) †	15.301	(15.702) †
(*d*) N_2 expired	4.534	4.534	15.702	15.702
(*e*) N_2 produced	0.130	(0) †	0.401	(0) †
(*f*) CO_2 produced	0.237	0.240	0.801	0.801
(*g*) O_2 consumed	0.266	0.302	0.970	1.060

* Extreme cases in these studies. All volumes are reduced to STPD.
† Assumed in the Haldane calculation (equation (1)).

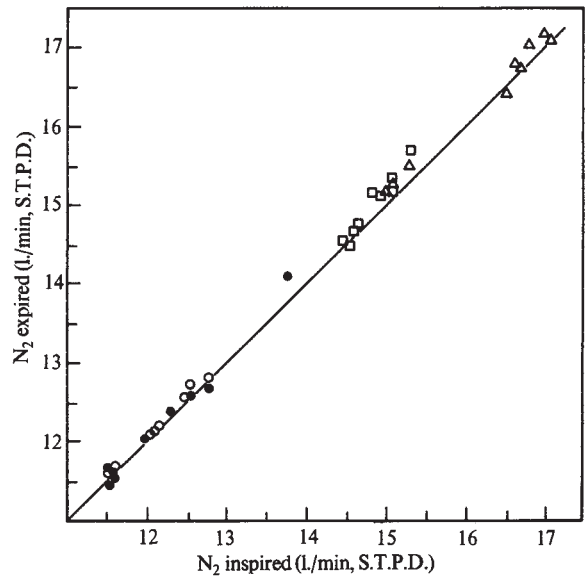

Fig. 1 Gaseous nitrogen inhaled and exhaled during walking on the level at 6 km/h. Four subjects were used, three men: L. D. (□); H. I. (△); J. P. (●), and one woman: S. P. (○). Values are in l./min STPD including calibration corrections. The line $y=x$ is for the case when the two values are equal, as assumed in the Haldane transformation. In actual experimentation, however, in thirty of thirty-five cases the nitrogen expired was greater than the nitrogen inspired. By χ^2 analysis, the probability of this difference being random is less than 0.001.

Our data suggest that the open circuit method is subject to hitherto disregarded sources of error. These may be accentuated in physical exercise. For the present argument, it is no matter whether the excess nitrogen is metabolic, gastrointestinal, bacteriological or dissolved in tissues—the effect on the calculations is the same. In some circumstances both the inspired and expired air may have to be measured directly.

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Patterns of Regeneration between Individual Nerve Cells in the Central Nervous System of the Leech

SEVERED axons of nerve cells can regrow towards their original destinations and re-establish synaptic connexions. For example, optic nerve fibres in the frog and goldfish grow back to the appropriate regions of the midbrain[1,2], and motor nerve cells in crayfish reinnervate their original muscles[3]. The new connexions seem to be correct, in the sense that normal function is restored by regenerating fibres growing back to the region that had been denervated. But it is not known how precisely an individual nerve cell can find its original target. One possibility is that each cell accurately reforms all its original synapses. On the other hand, the mechanism might be less precise, and the result still be functionally adequate or better, even though some cells have become reconnected abnormally. The general question of what happens at the cellular level during regeneration could also be relevant for an understanding of how specific neural connexions are formed during development.

In the experiments reported here, we have used the relatively simple central nervous system of the leech to study the regeneration of connexions between single, identified nerve cells, rather than large populations of neurones. The segmental ganglia in this animal are all similar, each containing only about 350 nerve cells. Earlier work has shown that fourteen sensory nerve cells can be identified reliably in ganglia from any animal[4]. Each of the cells has a characteristic set of properties by which it can be recognized, including (*a*) its shape and position in the ganglion, (*b*) the electrical properties of its membrane, (*c*) the specific type of stimulus to which it responds (touch, pressure or noxious mechanical stimuli), (*d*) the position of its receptive field and (*e*) the synaptic connexions that it makes with other cells in the same ganglion and in adjacent ganglia. The principle of the experiment is to cut one of the bundles of axons (a connective) that links two segmental ganglia, and see whether certain of these sensory cells become reconnected.

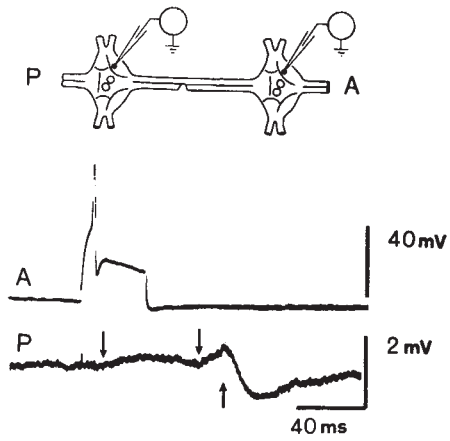

Fig. 1 Intracellular recordings from touch cells in adjacent ganglia of a normal leech. The arrangement for stimulating and recording is shown above. A single impulse in the touch cell in the anterior ganglion (A, upper trace) gives rise to two depolarizing synaptic potentials and a hyperpolarizing potential, indicated by arrows in the posterior touch cell (P, lower trace). Similar potentials were seen in the anterior cell following an impulse in the posterior cell. The cut in the lower connective disconnected the touch cells on that side of the two ganglia, so that stimulation of either cell no longer evoked synaptic potentials in the other.

In normal preparations which have not been operated on, the interconnexions of these sensory cells are specific and stereotyped[5]. Thus, the three touch cells on one side of one ganglion are connected to their homologues on the same side of each adjacent ganglion by way of fibres running through the ipsilateral connectives (Fig. 1). One or more impulses in any one of these three cells gives rise to complex synaptic potentials in all three touch cells in both of the adjacent ganglia. These connexions are "specific", in the sense that impulses occurring in touch cells do not evoke synaptic potentials in sensory cells of different modality or in many other cells that we can identify and have recorded from in adjacent ganglia. The traces in the lower part of Fig. 1 show the synaptic potentials evoked in a touch cell (P) after a touch cell (A) in the ganglion immediately anterior to it was stimulated by passing current through the intracellular microelectrode. The post-synaptic responses consist of depolarizing potentials, followed, after a variable delay, by a hyperpolarizing potential. Similar responses occur in touch cells in adjacent ganglia with stimulation in the opposite direction, that is, from posterior to anterior. All these interactions are disrupted when one cuts the connective on the same side as the sensory nerve cells. We have not yet located the cell bodies of any interneurones involved in the interactions of touch cells. Stimulation of a connective evokes hyperpolarizing and depolarizing synaptic potentials in touch cells.

To study regeneration, leeches were anaesthetized with 8% alcohol, and the connectives between a pair of ganglia exposed under the dissecting microscope; no one particular region of the animal was selected. One of the two connectives was cut completely. To be sure that the cut had severed the whole of the connective, it was always extended across the midline, so as to nick the other connective, which served to hold the two ends in approximate apposition. The skin was sutured with fine thread. After the leech had recovered from anaesthesia, a constriction of the body wall appeared at the site of the lesion and the swimming movements were abnormal: the two halves of the animal above and below the lesion no longer acted in