The Action of Leucogenenol on Human Lymphoblastoid and Murine Leukemic Cells in Tissue Culture[1] (35108)

F. A. H. RICE, M. L. BLUM, AND A. A. RENE[2]

The Department of Chemistry, American University, Washington, D.C. 20016

In an earlier communication Rice (1) reported the isolation from the metabolic products of *Penicillium gilmanii* of a compound, leucogenenol, that induced a leukocytosis in rabbits when as little as 0.001 μg/kg was injected intravenously. Rice and Shaikh (2) demonstrated by isolation that leucogenenol is normally present in bovine and human liver.

Leucogenenol gives no febrile response (3) and is not toxic when as much as 500 μg/g is injected into mice or 200 μg/kg into dogs or monkeys (4). Leucogenenol is not species specific (4) and its injection into animals induces a particularly noticeable increase in the number of myeloblasts and lymphoblasts found in the bone marrow and spleen (4). Injection of leucogenenol into mice whose myeloid and lymphoid tissues are partially destroyed by X-radiation causes the myeloid and lymphoid tissues to recover more rapidly than the tissues of untreated controls, suggesting that leucogenenol increases the rate of replication of myeloid and lymphoid cells (5). The stimulatory action of leucogenenol on the lymphoid cells of animals is also indicated by the fact that injection of leucogenenol into sublethally X-radiated mice decreases the latent period required for antibody formation (6).

The above results would be obtained if leucogenenol stimulated myeloid and lymphoid cells either directly or indirectly through the induced production of other compounds by the animal. To ascertain whether or not leucogenenol had a direct stimulatory action on cells, its effect on the respiratory quotient (RQ) and the rate of replication of cells in tissue culture was investigated. It was considered that if leucogenenol acted directly on cells it should affect the respiration and rate of replication of cells in tissue culture, whereas if its biological activity was due to the formation of other compounds by the animal it should have no effect on the respiration or growth of cells in tissue culture.

We report that the addition of 10^{-3} μg of leucogenenol to a growing culture of lymphoblastoid cells or murine leukemic cells increases the respiratory quotient as well as the rate of replication of the cells.

Materials and Methods. Normal lymphoblastoid cells of human origin (7, 8), Cee 8068 and Papermeister 1788F, were obtained through the courtesy of Dr. G. E. Moore, Roswell Park Memorial Institute, Buffalo, N.Y. The cells were maintained at 37° in not more than 20 ml of Eagle's minimum essential medium containing 20% fetal calf serum in tightly stoppered 125-ml Erlenmyer flasks in the presence of a mixture of 95% air and 5% CO_2. Eagle's Minimum Essential Medium[3] containing glutamine, but not bicarbonate, was obtained 10× concentrated and after the addition of the calculated quantity of fetal calf serum and bicarbonate, appropriate dilution was made to 2× with distilled water. Before use the medium was filtered under suction through a disposable Millipore filter[4] and the pH was adjusted to 7.2 with sterile CO_2 gas using phenol red as an internal

[1] This work was partially supported by The Office of Naval Research, contract No. N00013-67-C-0275 and by The Life Sciences Division of the U. S. Army Research Office under contract grant No. DA-ARO-49-092-65-686.

[2] Department of Experimental Pathology, Armed Forces Radiobiology Research Institute, Bethesda, Md.

[3] Procured from Grand Island Biological Co., Grand Island, N. Y.

[4] Obtained from A. H. Thomas Co., Washington, D. C.

indicator.

Cultures were started with approximately 2×10^5 cells/ml. When the population reached approximately 10^6 cells/ml, either aliquots of the culture were distributed into fresh medium to give approximately 2×10^5 cells/ml or the cells in the medium were collected by centrifugation at 1800 rpm for 10 min in a CS model International centrifuge and resuspended in fresh medium to give approximately 2×10^5 cells/ml. The usual aseptic techniques were used to handle the cells and medium.

Leukemic cells of mice (9) L-5178Y were obtained through the courtesy of Dr. G. A. Fischer, Department of Pharmacology, Yale University School of Medicine. The murine leukemic cells were grown on Fischer's medium[3] for leukemic cells of mice (10) with 10% horse serum added, otherwise the techniques were as used for the lymphoblastoid cells.

The replication of cells was followed by taking hemocytometer counts at 12-hr intervals. The leukemic cells of mice, since they grew in suspension, were counted after appropriate dilution and staining with 0.4% trypan blue, a vital exclusion dye, dissolved in Hanks' balanced salt solution.[3] The lymphoblastoid cells, however, grew in clumps and in order to obtain a uniform distribution the sample to be used for counting was diluted with 0.25% trypsin in phosphate buffer containing glucose[3] and allowed to stand at 37° for 30 min. Trypan blue was then added as before and the sample was counted. Duplicate counts were made and these were repeated if the two counts did not agree within 10%.

Media buffered with *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) were prepared by substituting equimolar quantities of HEPES for bicarbonate. The Eagle's minimum essential medium and the Fischer's medium for leukemic cells of mice were obtained in 10× concentration without bicarbonate and after the addition of the calculated quantity of HEPES and the fetal calf or horse serum, dilution was made in distilled water as previously described. The pH was 7.2.

The growth of each strain of cells in their appropriate medium buffered with HEPES was compared with their growth in the same medium buffered with bicarbonate. To do this, cells were collected by centrifugation at 1800 rpm from cultures maintained in the medium buffered with bicarbonate and a portion of these cells sufficient to yield a final concentration of approximately 2×10^6 cells/ml was resuspended in 20 ml of the bicarbonate buffered medium. A second portion of the cells sufficient to yield the same final concentration of 2×10^5 cells/ml was resuspended in 20 ml of the same medium buffered with HEPES instead of bicarbonate. At intervals of 12 hr the number of cells in each culture was determined by the use of a hemocytometer. At the end of the log phase of growth the cells in each of the two media were collected by centrifugation and resuspended in the same but fresh medium from which they were collected at the same initial concentration of 2×10^5 cells/ml. After three subcultures the cells growing in the medium buffered with HEPES were resuspended in a medium buffered with bicarbonate. Results obtained with Fischer's leukemic cells of mice are shown in Fig. 1. Essentially the same results as shown in Fig. 1 were obtained with the lymphoblastoid cells Cee 8068 and Papermeister 1788F. The above experiment was repeated with the modification that 10^{-2} μg of the calcium salt of leucogenenol was added to each medium. The results were essentially as shown in Fig. 1; however, an increased rate of growth of the cells was observed in the media that contained leucogenenol. The effect of adding leucogenenol to a medium buffered with bicarbonate is shown in Fig. 2.

To provide the cells for the respirometer studies, cells were grown in approximately 25 ml of the appropriate medium buffered with bicarbonate contained in 200 ml BellCo spinner flasks.[5] The cells were harvested in the log phase of growth by centrifugation at 1800 rpm and then resuspended in the medium appropriate for the growth of that particular cell. The medium was buffered with HEPES. Aliquots of the suspension of cells were then transferred to the respirometer flasks.

[5] Manufactured by BellCo Glass Co., Vineland, N.J.

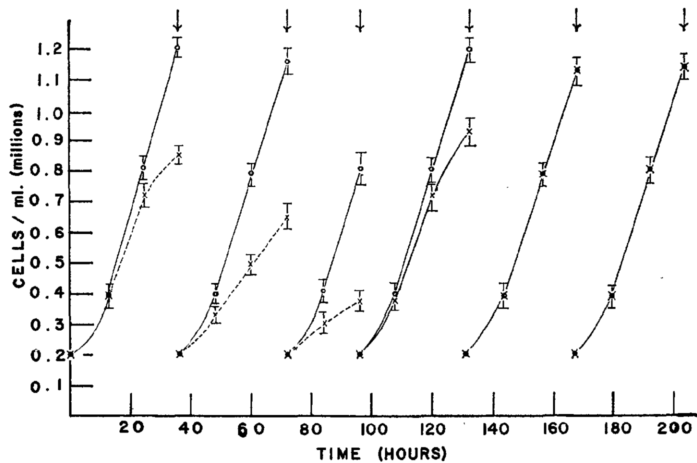

FIG. 1. Effect on the growth of Cee 8068 lymphoblastoid cells of using *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) as the buffering agent. For details of the medium see text. Cells were subcultured at times indicated by ↓. (—) bicarbonate buffer; (- - -) HEPES buffer; (O), cells in bicarbonate buffer only; (×), cells in HEPES buffer followed by bicarbonate buffer after three subcultures. Vertical bars represent the standard deviations calculated from a minimum of 6 experiments.

The Warburg apparatus used was a circular refrigerated model manufactured by the Precision Scientific Co., Chicago, Ill. modified so that the flasks oscillated at 60 cycles/min. The bath temperature was maintained at 37 ± 0.01°. Respirometer flasks were grouped in sets of three around the Warburg apparatus. Each flask contained in the center well the same size piece of fluted filter paper. The center flask of the three was the thermobarometer and contained 3.1 ml of medium. To the flasks on each side of the thermobarometer was added 2.9 ml of medium containing approximately 3×10^6 cells and 0.1 ml of either pyrogen-free sterile isotonic saline or pyrogen-free sterile isotonic saline containing 10^{-3} μg of the calcium salt of leucogenol. In one of the flasks at the side

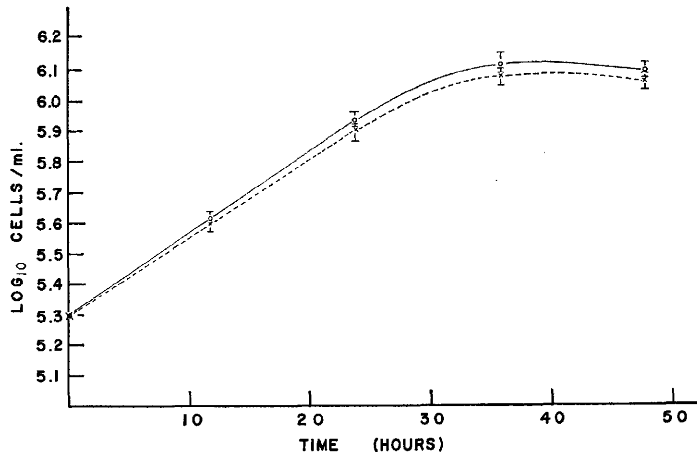

FIG. 2. Effect of adding 10^{-3} μg of the calcium salt of leucogenenol to a culture of lymphoblastoid cells Cee 8068, growing in a medium buffered with bicarbonate at pH 7.2. For details of the medium see text. (- - -) medium without added leucogenenol; (—) medium with 10^{-3} μg of leucogenenol added. Vertical bars represent the standard deviations calculated from a minimum of 6 experiments.

of the thermobarometer the filter paper was saturated with 0.1 ml of 6 *N* KOH to absorb the liberated CO_2. Respirometer flasks were calibrated by the use of hydrazine sulfate and $K_3Fe(CN)_6$, and the calibrations were checked by the use of mercury (11). Manometers were read at 30-min intervals and calculations were made by standard methods (11).

Results and Discussion. In order to avoid the difficulties associated with differentiating between CO_2 liberated by the cells and CO_2 liberated by changes in the medium, such as pH, it was desirable to determine respiratory quotients in a medium that was not buffered with bicarbonate. The possibility therefore of substituting *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) for bicarbonate was investigated. Figure 1 shows the results obtained when cells were grown on a medium buffered with HEPES in contrast to the results obtained when the same cells were grown on the same medium buffered with bicarbonate. As shown, the growth rate for the first 12 hr was the same in the HEPES as in the bicarbonate buffered medium. However, after approximately 12 hr the cells in the medium buffered with HEPES grew at an appreciably slower rate than the same cells in the medium buffered with bicarbonate. Repeated culture in the medium buffered with HEPES further decreased the rate of growth of the cells. However, when the cells that had been cultivated in the medium buffered with HEPES were cultivated on a medium buffered with bicarbonate they regained their original rate of growth (Fig. 1). The first time the cells were cultured on a medium buffered with HEPES their rate of growth was the same for 12 hr as when they were cultured on a medium buffered with bicarbonate. Hence it was considered that during this 12-hr period the measurement of the respiratory quotient and any change in the respiratory quotient caused by the addition of leucogenenol to the medium would be accurate to at least a first approximation. Accordingly respirometer studies were carried out in media buffered with HEPES.

TABLE I. Experiments Showing the Change in the Respiratory Quotient (RQ) and Oxygen Consumption Produced by the Addition of 10^{-3} μg of Leucogenenol to Cultures of 1.2 to 3.3 $\times$ 10^6 Cee 8068 Lymphoblastoid Cells Growing in Approximately 3 ml of Eagle's Minimum Essential Medium[a] Containing 20% Fetal Calf Serum[a] and Buffered with *N*-2-Hydroxyethylpiperazine-*N'*-ethanesulfonic acid[b] (HEPES).

Expt. no.	RQ	O_2 consumption (μl of $O_2/10^6$ cells/hr)	No. of cells (millions)
Without the addition of leucogenenol to the medium			
21	0.86	1.96	2.00
22	0.793	1.92	1.20
23	0.837	1.82	3.10
24	0.842	1.98	2.80
25	0.862	1.94	3.30
26	0.82	1.90	2.59
27	0.943	2.04	2.97
Av	0.851 $\pm$ 0.05	1.90 $\pm$ 0.07	2.61 $\pm$ 0.74
With the addition of 10^{-3} μg of leucogenenol to the medium			
28	1.17	1.58	2.93
29	1.15	1.73	2.80
30	1.03	1.59	2.90
31	1.16	1.40	3.00
32	1.22	1.78	3.00
33	1.06	1.52	3.21
Av	1.13 $\pm$ 0.03	1.61 $\pm$ 0.14	2.97 $\pm$ 0.14

[a] Obtained from Grand Island Biological Co., Grand Island, N. Y.

[b] Obtained from Calbiochem, Los Angeles, Calif. For details of the medium see text.

Table I shows the effect on the RQ and oxygen consumption of adding leucogenenol to the medium in which Cee 8068 lymphoblastoid cells were growing. The number of cells between 1.2 $\times$ 10^6 cells/ml and 3.3 $\times$ 10^6 cells/ml did not alter the respiratory quotient or oxygen consumption within the limits of experimental error. The RQ of the Cee 8068 lymphoblastoid cells was 0.85 $\pm$ 0.05 and the oxygen consumption was 1.90 $\pm$ 0.07 μl of $O_2/10^6$ cells/hr. The addition of leucogenenol to the medium increased the RQ to 1.13 $\pm$ 0.03 ($p < 0.001$ for the difference) and decreased the oxygen consumption to 1.61 $\pm$ 0.14 μl $O_2/10^6$ cells/hr ($p < 0.001$ for the difference). Similar results were obtained with the two other strains of cells

TABLE II. The Effect on the Respiratory Quotient (RQ) and Oxygen Consumption of Adding 10^{-3} μg of Leucogenenol to Three Types of Cells Growing in Approximately 3 ml of Medium Buffered with N-2-Hydroxyethylpiperazine-N′-ethanesulfonic Acid[a] (HEPES).

	RQ		O_2 consumption (μl of $O_2/10^6$ cells/hr)		No. of cells (millions)	
Cell type	Without leucogenenol	With leucogenenol	Without leucogenenol	With leucogenenol	Without leucogenenol	With leucogenenol
Cee 8068[b]	0.85 ± 0.05	1.13 ± 0.03	1.90 ± 0.07	1.61 ± 0.14	2.61 ± 0.74	2.97 ± 0.14
Papermeister 1788F[b]	0.80 ± 0.02	1.01 ± 0.03	1.80 ± 0.08	1.63 ± 0.12	3.00 ± 0.10	2.82 ± 0.40
Fischer L-5178Y[c]	1.02 ± 0.05	1.71 ± 0.35	1.80 ± 0.49	2.12 ± 0.18	3.00 ± 0.10	3.00 ± 0.10

[a] Obtained from Calbiochem, Los Angeles, Calif.

[b] In Eagle's minimum essential medium containing 20% fetal calf serum and buffered with HEPES. For details see text.

[c] In Fischer's medium for leukemic cells of mice containing 10% horse serum and buffered with HEPES. For details see text.

investigated (Table II). It is of interest that while the RQ and oxygen consumption of the two strains of lymphoblastoid cells were essentially the same (Table II), the RQ and oxygen consumption of the leukemic cells of mice were quite different from that of the lymphoblastoid cells. This suggests the possibility that leucogenenol might have a significantly different activity on different cell strains.

Figure 2 shows the action of leucogenenol on cell replication. There is an increase in the rate of growth when leucogenenol is present in the medium. After 36 hr there is also a 10% increase in the number of cells over that found in the medium to which no leucogenenol had been added. Similar results (not shown) were obtained in media buffered with HEPES. However, the addition of leucogenenol to the media buffered with HEPES gave only a 2.5% increase in the number of cells after 36 hr.

It would certainly appear that leucogenenol affects directly both the respiratory quotient and rate of replication of cells growing in tissue culture and therefore in all probability acts directly on the blood cells of animals.

The finding that leucogenenol is a normal constituent of liver tissue (2), together with its associated biological activity, suggests that it normally plays a role in the growth and regulation of the number of blood cells in the body.

Summary. It has been found that the addition of leucogenenol to a medium in which lymphoblastoid or leukemic cells of mice are growing increases the respiratory quotient and the rate of replication of the cells. It would appear therefore that leucogenenol's known activity in the animal is due to a direct action of leucogenenol on the blood cells.

The fact that leucogenenol is normally present in liver tissue suggests that leucogenenol plays a role in the normal growth and regulation of blood cells in the body.

We thank Dr. G. E. Moore, Roswell Park Memorial Institute and Dr. G. A. Fischer, Department of Pharmacology, Yale University Medical School, for the cultures of lymphoblastoid and leukemic cells of mice, respectively. We also thank Mrs. Margaret M. Rice for assistance in preparing the manuscript.

1. Rice, F. A. H., Proc. Soc. Exp. Biol. Med. **123**, 189 (1966).
2. Rice, F. A. H., and Shaikh, B., Biochem. J. **116**, 709 (1970).
3. Rice, F. A. H., J. Infec. Dis. **118**, 76 (1968).
4. Rice, F. A. H., and Darden, J. H., J. Infec. Dis. **118**, 289 (1968).
5. Rice, F. A. H., Lepick, J., and Darden, J. H., Radiat. Res. **36**, 144 (1968).
6. Rice, F. A. H., Lepick, J., and Hepner, P., Radiat. Res. **42**, 164 (1970).
7. Moore, G. E., J. Surg. Res. **9**, 139 (1969).
8. Moore, G. E., and McLimans, W. F., J. Theor. Biol. **20**, 217 (1968).

9. Fischer, G. A., Ann. N. Y. Acad. Sci. **76**, 673 (1958).

10. Fischer, G. A., and Sartorelli, A. S., Methods Med. Res. **10** (1964).

11. Umbreit, W. W., Burris, R. H., and Stauffer, I. P., "Manometric Techniques." Burgess, Minneapolis (1957).

Received April 7, 1970. P.S.E.B.M., 1970, Vol. 135.