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# EFFECTS OF VINCALEUKOBLASTINE SULFATE ON METABOLISM OF THIOGUANINE-RESISTANT L1210 LEUKEMIA CELLS

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**Abstract**—Stimulation of aerobic acid production with 1 ppm vincaleukoblastine sulfate or 50 ppm leurocristine sulfate without inhibition of respiration was observed with thioguanine-resistant L1210 leukemia cells. To obtain a significant inhibition of respiration in time, at least 25 ppm vincaleukoblastine was required. Addition of glucose inhibited respiration of these leukemia cells (Crabtree effect) without affecting the per cent respiratory inhibition by vincaleukoblastine. Anaerobic glycolysis was not affected by 1 ppm of the alkaloid but was markedly inhibited (up to 90%) by 50 ppm. Respiratory and glycolytic inhibition by vincaleukoblastine at the concentrations studied was not affected by the absence of externally added inorganic phosphate. Monosodium glutamate (0·01 M) reduced the effect of vincaleukoblastine on aerobic acid production in line with reports of glutamic acid protection against this alkaloid with leukemia cells in tissue culture and Ehrlich ascites cells *in vivo*. The observations presented provide evidence that both vincaleukoblastine and leurocristine cause an inhibition of the Pasteur effect. This action of these alkaloids may affect cell energy production required for mitosis.

VINCALEUKOBLASTINE (VLB),* an alkaloid isolated from the periwinkle plant (*Vinca rosea* Linn.), produces C-mitosis (metaphase arrest) in dividing cells.[1, 2] The origin, isolation, and physical chemistry of this and related compounds have been thoroughly described by a team of investigators from the Lilly Research Laboratories.[3–5] VLB has been reported to be effective against rodent cancers,[2, 6, 7] tissue cultures,[6, 8] and some trophoblastic tumors in women that have developed resistance to methotrexate.[9] Cell division requires energy, and there is strong evidence for the view that dividing cells derive their energy from oxidative phosphorylations and carbohydrate metabolism (see p. 171, Ref. 10). There is some evidence that cells build up the energy reserve required for cell division during interphase (Ref. 10, p. 179). It has been shown by the experiments of Mazia and Prescott with amoebae[11] that $^{32}P$ uptake increases during interphase to twice its uptake during cell division. The phosphorus taken up during interphase could be used in oxidative phosphorylation as well as in nucleic acid synthesis (Ref. 10, p. 178). The work of Bullough[12] has indicated that the presence of oxygen and glucose is necessary for the actual beginning of mitosis. Since VLB causes C-mitotic arrest, it seemed possible that the alkaloid might produce some derangement in the energy metabolism of the cell. The present study describes the

* The author is indebted to Miss Dorothy G. Walker, 4th Surgical Division, Bellevue Medical Center, New York, N.Y., and to Dr. Otto K. Behrens of Lilly Research Laboratories, Indianapolis, Ind., for the vincaleukoblastine sulfate (Velban) used in these studies.

effects of VLB on aerobic and anaerobic metabolism of glucose in cells of a thioguanine-resistant subline of the L1210 mouse leukemia.

## MATERIALS AND METHODS

The thioguanine-resistant[13] subline of the L1210 leukemia as ascites was obtained through the generosity of Mr. Donald Foor of the National Cancer Institute, Bethesda, and was maintained by serial passage in BALB/c × DBA/2 ($F_1$) mice of both sexes. The Krebs-2 and Ehrlich carcinoma ascites were also maintained by serial passage in BALB/c × DBA/2 ($F_1$) mice of both sexes.

Analyses of cell metabolism were performed by the Warburg technique. Aerobic conditions were provided by aerating with a mixture of 5% $CO_2$ in air for glycolytic studies and by shaking in air alone for measurements of respiratory activity. Anaerobic conditions were provided by gassing with a mixture of 5% $CO_2$ in nitrogen which had been passed, together with a small amount of hydrogen (about 1%), over hot copper to remove traces of oxygen. Acid production was measured in terms of $CO_2$ released from the bicarbonate-buffered medium; pH determinations were frequently made of the vessel contents when an experiment was completed. Respiration was measured by the Burk modification[14] of the Warburg-Krippahl method[15] and by the classical KOH method.[16] Both methods gave identical results, but the former was much more convenient to use and required no reduction in the level of sodium bicarbonate in the medium, thus allowing the measurement of glycolysis and respiration under identical conditions of carbon dioxide pressure and of bicarbonate concentration. Incubation temperature was 37° unless otherwise stated. Glucose disappearance was measured by the glucostat* method.[17, 18] Neither the Krebs-Ringer medium nor the concentrations of VLB used interfered with the colorimetric determination of glucose.

The medium used was Krebs-Ringer-bicarbonate[14] to which glucose was added. Glucose concentration in the medium was usually $1{\cdot}1 \times 10^{-2}$ M and $NaHCO_3$ concentration was $3{\cdot}6 \times 10^{-2}$ M except when measurements were made of glucose disappearance. In such latter cases, glucose concentration was $5{\cdot}6 \times 10^{-3}$ M and $NaHCO_3$ concentration was $2{\cdot}4 \times 10^{-2}$ M. When the KOH method was used to measure respiration, $8{\cdot}9 \times 10^{-3}$ M $NaHCO_3$ was used. Vincaleukoblastine as sulfate was added to the tumor cell suspension in the Warburg flask to give a final concentration of from 1 to 100 ppm or $1{\cdot}1 \times 10^{-6}$ to $1{\cdot}1 \times 10^{-4}$ M. At these concentrations VLB did not influence the pH of the incubation medium. Initial dry weight was calculated as 16% of the packed cell volume obtained by centrifuging the cells at a relative centrifugal force of 900 *g* for 20 min.

The following symbols have been used to designate the metabolic activities measured: $Q_{O_2}$ = microliters of oxygen consumed per milligram initial dry weight per hour. For this study, $Q^{air}_{CO_2}$ represents microliters of $CO_2$ produced per milligram initial dry weight per hour in air and in excess of concurrent respiration. $Q^{N_2}_{CO_2}$ = microliters of $CO_2$ produced per milligram initial dry weight per hour under anaerobic conditions. Q values are based on the average of measurements in microliters obtained from duplicate manometric determinations. Glucose determinations were performed on supernatants of the pooled contents of duplicate vessels. Each figure and table

* Worthington Biochemical Corp. preparation of glucose oxidase and chromogenic hydrogen donor suitable for the rapid, quantitative, colorimetric determination of glucose.

presents the data obtained from representative experiments. The results are reproducible, and each experiment was internally controlled.

## RESULTS

In preliminary studies, cells of several mouse ascites cancers suspended in Krebs-Ringer-bicarbonate medium were exposed to concentrations of VLB from 1 to 100 ppm. The gas phase was air with 5% carbon dioxide. These preliminary studies were designed to take advantage of the fact, first discovered by Warburg,[19] that most, even if not all, cancer cells produce lactic acid from glucose under aerobic conditions. It was reasoned that an effect of VLB on this metabolic parameter would indicate that some facet or facets of the energy-providing processes of the cell were involved in the action of this drug. Incubation with 50 ppm VLB *in vitro* caused up to 35% stimulation of aerobic acid production in Ehrlich carcinoma and 8-azaguanine-resistant L1210 leukemia, and 80 to 100% stimulation in thioguanine-resistant L1210 leukemia ascites cells, but caused up to 25% inhibition of this metabolic parameter in cells of the parent L1210 line.[20] Although aerobic and anaerobic metabolism of all leukemia cells studied was affected by 50 ppm VLB,[20] only the thioguanine-resistant L1210 leukemia cells responded to 1 ppm VLB, and then in only one metabolic parameter—*i.e.* aerobic acid production from glucose. The thioguanine-resistant L1210, because of its sensitivity to VLB and its high yield of tumor cells with practically no red blood

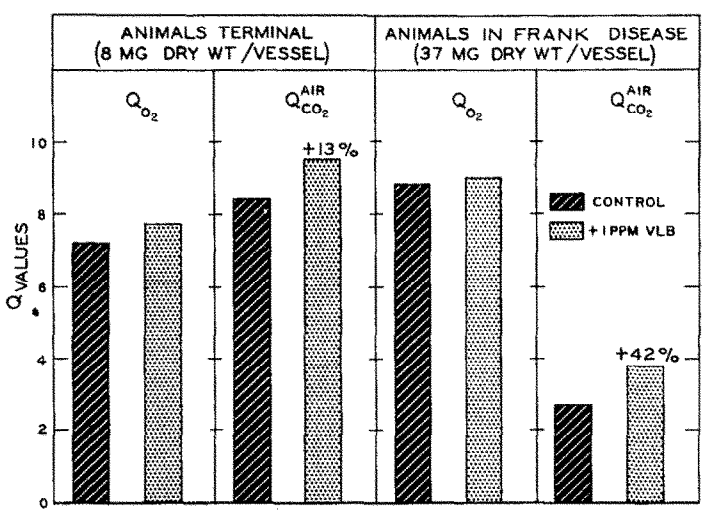


FIG. 1. Effect of 1 ppm (1 mg/L) vincaleukoblastine sulfate on aerobic metabolism of glucose by thioguanine-resistant L1210 leukemia cells after 30-min incubation at 37°.

cell contamination (in marked contrast to other L1210 leukemias), made it a desirable choice for detailed study of metabolic effects of the drug. Fig. 1 shows the effect of 1 ppm VLB on aerobic metabolism of glucose by thioguanine-resistant L1210 cells after 30-min incubation at 37°. In the representative experiment presented, metabolic effects of VLB on cells from animals in terminal disease and from animals in frank

disease* were measured. With cells from animals in both disease states, stimulation of aerobic acid production in the presence of VLB was observed, although the greater per cent effect was measured in cells from the animals in frank disease. While the per cent stimulation of aerobic acid production by VLB was small in cells from animals in terminal disease, it should be noted that the control $Q\overset{air}{CO_2}$ (8·4) of the "terminal" cancer cells was three times as high as the $Q\overset{air}{CO_2}$ of the cancer cells from animals in frank disease (2·7). The high $Q\overset{air}{CO_2}$ of the control "terminal" cancer cells suggests that the Pasteur-effect mechanism in these cells is less effective than it is in cancer cells from animals in frank disease. In such a situation it is quite possible that an effect of VLB resulting in inhibition of the Pasteur effect could be masked by the diminution of the Pasteur effect due to aging in the "terminal" cancer cells. The higher aerobic acid

TABLE 1. SUMMARY OF EXPERIMENTS SHOWING THE EFFECT OF VINCALEUKOBLASTINE ON $Q\overset{air}{CO_2}$ OF THREE ASCITES TUMORS

| Expt. | Control | + 1 ppm VLB | Stimulation (%) | + 50 ppm VLB | Stimulation (%) |
|---|---|---|---|---|---|
| | | L1210 (thioguanine-resistant) animals in frank disease | | | |
| 1 | 2·6 | 3·8 | 46 | | |
| | 2·8 | 3·8 | 36 | | |
| 2 | 3·1 | 4·3 | 39 | 6·6 | 113 |
| 3 | 1·3 | 2·3 | 77 | | |
| | 1·5 | 2·4 | 60 | | |
| 4 | 2·4 | 3·7 | 54 | | |
| 5 | 2·8 | 4·3 | 54 | 5·5 | 96 |
| | 2·6 | 4·1 | 58 | 4·9 | 88 |
| 6 | 1·5 | 2·6 | 73 | | |
| | 1·6 | 2·8 | 75 | | |
| | | L1210 (thioguanine-resistant) animals in terminal disease | | | |
| 7 | 8·2 | 9·5 | 16 | 10·4 | 27 |
| | 8·6 | 9·5 | 10 | 10·6 | 23 |
| | | Krebs-2 | | | |
| 8 | 22·2 | | | 24·1 | 8·6 |
| | 22·6 | | | 24·5 | 8·4 |
| | | Ehrlich | | | |
| 9 | 20·6 | | | 28·1 | 36 |
| | 21·6 | | | 27·2 | 26 |

production in cells from the animals with terminal disease is in line with the findings of Levy *et al.*[21] that, in general, the older the tumor the higher the aerobic glycolysis. On the other hand, respiration was somewhat lower in cells from terminal animals, suggesting a shift to a more glycolytic metabolism. A summary of experiments showing the effect of VLB on $Q\overset{air}{CO_2}$ of three ascites tumors is presented in Table 1.

* Animals were considered to be in frank disease when they were obviously distended with ascites but were not moribund, and to be in terminal disease when they were quite inactive with a life expectancy of less than 24 hr.

Table 2 compares the effect of VLB on glucose utilization of thioguanine-resistant L1210 leukemia cells and Krebs-2 carcinoma cells. The data show that, with thioguanine-resistant L1210 cells, glucose disappearance in the presence of VLB parallels increased $Q\overset{air}{CO_2}$ at 1 and 50 ppm. Krebs-2 cells, which showed a small manometric response to VLB, showed a small stimulation of glucose utilization in the presence of 50 ppm VLB. Final pH measurements were consistent with the manometric and glucose measurements.

TABLE 2. EFFECT OF VINCALEUKOBLASTINE ON GLUCOSE UTILIZATION *IN VITRO* OF TWO ASCITES TUMORS*

| Tumor | Control | +1 ppm VLB | Change (%) | +50 ppm VLB | Change (%) |
|---|---|---|---|---|---|
| | | $Q\overset{air}{CO_2}$ (Incubation time = 1 hr) | | | |
| Thioguanine-resistant L1210 | 2·7 | 4·2 | +56 | 5·2 | +93 |
| | | Glucose used (= $\mu$l $CO_2$ equivalent to acid formed) | | | |
| | 87·5† | 125 | +43 | 180 | +105 |
| Final pH | 7·40 | 7·30 | | 7·25 | |
| | | $Q\overset{air}{CO_2}$ (Incubation time = 2 hr) | | | |
| KREBS-2 carcinoma | 22·4 | | | 24·3 | +8·5 |
| | | Glucose used (= $\mu$l $CO_2$ equivalent to acid formed) | | | |
| | 402‡ | | | 440 | +9·0 |
| Final pH | 7·18 | | | 7·15 | |

* Krebs-Ringer, $5{\cdot}6 \times 10^{-3}$ M glucose, $2{\cdot}4 \times 10^{-2}$ M $NaHCO_3$, average hourly rates (1 $\mu$M glucose = 44·8 $\mu$l $CO_2$ as lactic acid).
† 1·95 $\mu$M glucose.
‡ 8·97 $\mu$M glucose.

Fifty ppm VLB caused up to 50% inhibition of respiration (Table 3) in thioguanine-resistant cells after incubation for 2 hr. Although the addition of glucose to the incubation medium resulted in an inhibition of cell respiration (Crabtree effect), the per cent respiratory inhibition by VLB was not affected (Fig. 2). Omission of inorganic phos-

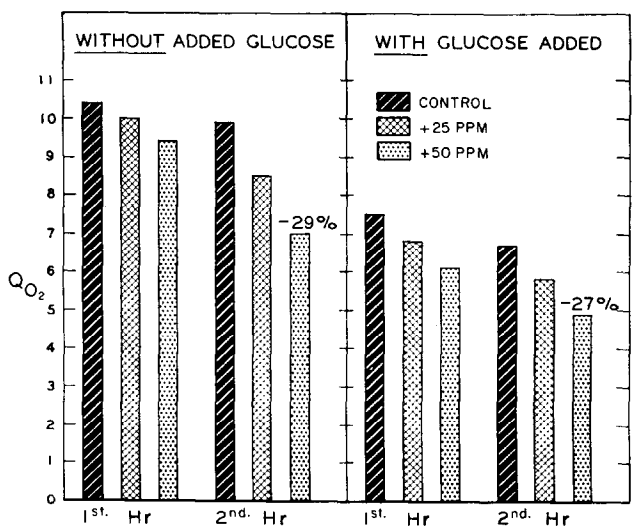


FIG. 2. Effect of vincaleukoblastine on respiration of thioguanine-resistant L1210 leukemia cells.

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TABLE 3. EFFECT OF ABSENCE OF INORGANIC PHOSPHATE FROM THE INCUBATION MEDIUM ON INHIBITION OF GLYCOLYSIS AND RESPIRATION OF THIOGUANINE-RESISTANT L1210 LEUKEMIA CELLS BY VINCALEUKOBLASTINE

| Time | With phosphate | | | | | Without phosphate | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | +1 ppm VLB | Effect (%) | +50 ppm VLB | Effect (%) | Control | +1 ppm VLB | Effect (%) | +50 ppm VLB | Effect (%) |
| | | | | | Anaerobic glycolysis ($Q\overset{N_2}{CO_2}$)* | | | | | |
| First hour | 23·4 | 23·3 | −0·4 | 3·0 | −87 | 21·6 | 21·6 | 0 | 2·1 | −90 |
| Second hour | 20·6 | 21·1 | +2 | 1·3 | −94 | 19·7 | 19·7 | 0 | 0·9 | −95 |
| | | | | | Respiration ($Q_{O_2}$)† | | | | | |
| First hour | 3·8 | 4·0 | +5 | 3·2 | −16 | 2·7 | 3·2 | +19 | 2·2 | −19 |
| Second hour | 3·2 | 3·0 | −6 | 1·5 | −53 | 2·0 | 1·8 | −10 | 1·0 | −50 |
| Third hour | 2·3 | 2·2 | −4 | 1·0 | −57 | 1·3 | 1·3 | 0 | 0·5 | −62 |

* Krebs-Ringer, $1·1 \times 10^{-2}$ M glucose, $3 \times 10^{-2}$ M $NaHCO_3$, 7 mg calculated dry weight, average hourly rates.

† Same medium and incubation temperature but 6 mg calculated dry weight, average hourly rates.

phate from the medium did not alter the VLB-induced inhibition of respiration and anaerobic glycolysis of thioguanine-resistant cells at the drug concentrations studied (Table 3). Other studies from this laboratory have shown that the absence of extracellular inorganic phosphate markedly increased inhibition of glycolysis in Ehrlich and Krebs-2 ascites cells by 5-fluorouracil or 5-fluorouridine.[22] A similar increase in glycolytic inhibition was obtained with S91 melanoma slices and methotrexate[23] in a phosphate-free medium.

Thioguanine-resistant cells were incubated with VLB in the presence and absence of $1 \times 10^{-2}$ M monosodium glutamate (Fig. 3). Stimulation of aerobic acid production

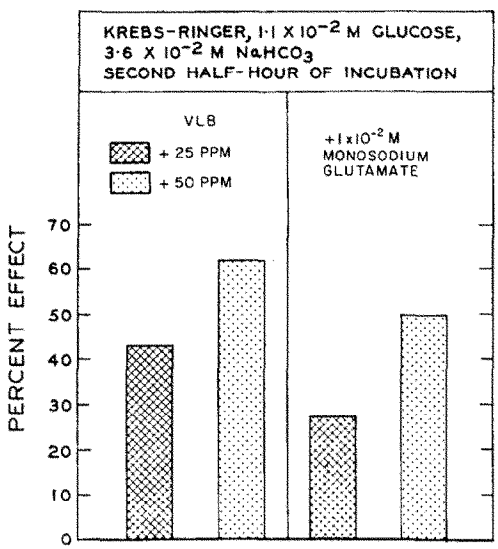


FIG. 3. Per cent stimulation of aerobic acid production by vincaleukoblastine in the presence and absence of monosodium glutamate. Krebs-Ringer medium, $1{\cdot}1 \times 10^{-2}$ M glucose, $3{\cdot}6 \times 10^{-2}$ M $NaHCO_3$; percentages based on the average 30-min rate during the second half hour of incubation relative to controls.

in these cells above the control level by 25 and 50 ppm VLB was less in the presence of monosodium glutamate than in its absence. Observed effects of monosodium glutamate with thioguanine-resistant leukemia cells are in line with reports of glutamic acid protection against VLB by Johnson *et al.*[6] in studies with leukemia cells in tissue culture and by Cutts *et al.*[7] in studies with Ehrlich ascites cancers *in vivo*. Of the amino acids, glutamate occupies a special position, in that it can be oxidized to carbon dioxide and water after oxidative deamination to α-ketoglutaric acid, without the presence of other metabolites.[24] Because of this special property, glutamate may be able to compensate to some degree for the VLB-induced lesion in oxidative metabolism which apparently involves the Pasteur effect.

It was of interest to compare the effects of VLB and the related alkaloid, leurocristine (VCR)* on aerobic metabolism of thioguanine-resistant L1210 cells. Cells

* Leurocristine used for this study was obtained through the kindness of Dr. Emil Frei, III, National Cancer Institute.

from animals in frank disease were suspended in standard Krebs-Ringer medium and incubated at 33°. Fifty ppm of either VCR (about $5{\cdot}3 \times 10^{-5}$ M) or VLB stimulated aerobic glycolysis to the same extent. However, inhibition of respiration by VCR was not significant even after 4 hr. Final pH measurements were consistent with the stimulatory effects of VLB and VCR on aerobic acid production (Table 4). It was found

TABLE 4. EFFECT OF VINCALEUKOBLASTINE AND LEUROCRISTINE ON AEROBIC ACID PRODUCTION AND RESPIRATION OF THIOGUANINE-RESISTANT L1210 LEUKEMIA CELLS

| Time | Control | +50 ppm VLB | Effect (%) | +50 ppm VCR | Effect (%) |
|---|---|---|---|---|---|
| | | Aerobic acid production ($Q\overset{air}{CO_2}$)* | | | |
| Last 40 min of first hour | 1·9 | 3·1 | +63 | 3·0 | +58 |
| pH (after 2 hr) | 7·2 | 7·1 | | 7·1 | |
| | | Respiration ($Q_{O_2}$)* | | | |
| Last 50 min of first hour | 5·4 | 5·0 | −7 | 5·4 | 0 |
| Second hr | 5·7 | 4·8 | −16 | 5·3 | −7 |
| Third hr | 5·6 | 4·4 | −21 | 5·2 | −7 |
| Fourth hr | 5·9 | 4·7 | −20 | 5·4 | −8 |

* Krebs-Ringer, $1{\cdot}1 \times 10^{-2}$ M glucose, $3 \times 10^{-2}$ M $NaHCO_3$, incubation temperature = 33°, 18·9 mg calculated dry weight, average hourly rates.

in this experiment that although the metabolic rate of the cells was decreased, probably because of the lower incubation temperature, the effect of VLB and VCR on aerobic acid production was apparently not affected.

## DISCUSSION

Pasteur,[25] was first to observe that the presence of oxygen decreases the fermentation of sugar by living organisms (The Pasteur effect). [26–28] It was shown by Pasteur that the weight of sugar consumed in fermentation is completely out of proportion to the weight of yeast substance formed (ratio 176 to 1), and that in the presence of air, carbohydrate utilization in proportion to the weight of yeast formed is not so marked (ratio 4 to 1). The presence of an incomplete Pasteur effect in yeast cells or in cancer cells is indicative of an aberration in the mechanism(s) by which glycolysis and respiration control each other. When the Pasteur effect is diminished in tumors or normal tissues, an increased aerobic glycolysis results which may or may not be accompanied by inhibition of respiration.[26] One of the conditions under which the Pasteur effect becomes deficient or inhibited is the addition in low concentrations of certain abnormal substances to the medium bathing the cell. Many of these substances simultaneously cause a rise in respiration but most, at a higher concentration, also inhibit respiration.[26] In the present study, a marked stimulation of aerobic acid production with 1 ppm VLB and 50 ppm VCR, without significant inhibition of respiration,

has been observed with thioguanine-resistant L1210 leukemia cells from animals in frank disease. To obtain a significant inhibition of respiration in time, at least 25 ppm VLB was required. Fifty ppm VLB, in addition to producing 80 to 100% stimulation of aerobic acid production from glucose, caused up to 95% inhibition of anaerobic glycolysis; only one-fiftieth as much VLB caused 40 to 60% stimulation of aerobic acid production from glucose with no effect on anaerobic glycolysis. The results obtained provide evidence that both vincaleukoblastine and leurocristine inhibit the Pasteur effect. This action of these alkaloids may affect cell energy production required for mitosis. That a critical level of energy reserve is necessary for complete mitosis is indicated by the observations of Bullough[12] in mammary carcinomas that new prophases can be formed up to 2 hr after stoppage of blood supply without subsequent completion of mitotic division.

## REFERENCES


1. J. H. Cutts, *Cancer Res.* **21**, 168 (1961).
2. G. Cardinali, G. Cardinali and J. Blair, *Cancer Res.* **21**, 1542 (1961).
3. G. H. Svoboda, N. Neuss and M. Gorman, *J. Amer. pharm. Ass.* **48**, 659 (1959).
4. N. Neuss, M. Gorman, G. H. Svoboda, G. Maciak and C. T. Beer, *J. Amer. chem. Soc.* **81**, 4754 (1959).
5. M. Gorman, N. Neuss and G. H. Svoboda, *J. Amer. chem. Soc.* **81**, 4745 (1959).
6. I. S. Johnson, H. F. Wright, G. H. Svoboda and J. Vlantis, *Cancer Res.* **20**, 1016 (1960).
7. J. H. Cutts, C. T. Beer and R. L. Noble, *Cancer Res.* **20**, 1023 (1960).
8. D. G. Walker and J. C. Wright, *Cancer Chemother. Reports* **14**, 139 (1961).
9. R. Hertz, M. B. Lipsett and R. H. Moy, *Cancer Res.* **20**, 1050 (1960).
10. J. Brachet, *Biochemical Cytology*. Academic Press, New York (1957).
11. D. Mazia and D. M. Prescott, *Science* **120**, 120 (1954).
12. W. S. Bullough, *Exp. Cell Res.* **1**, 410 (1950).
13. L. W. Law, *Ann. N.Y. Acad. Sci.* **71**, 976 (1958).
14. D. Burk, *Ann. N.Y. Acad. Sci.* **92**, 372 (1961).
15. O. Warburg and G. Krippahl, *J. nat. Cancer Inst.* **24**, 51 (1960).
16. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques*. Burgess, Minneapolis (1957).
17. D. Keilin and E. F. Hartree, *Biochem. J.* **42**, 230 (1948).
18. A. S. Keston, in *Abstracts of Papers*, 130th Meeting, Amer. Chem. Soc., p. 69C, Sept. 1956.
19. O. Warburg, *The Metabolism of Tumors*. Constable, London (1930).
20. J. C. Hunter, in *Abstracts*, VIIIth International Cancer Congress, p. 536, Moscow (1962).
21. H. B. Levy, H. Davidson, R. Reinhart and A. L. Schade, *Cancer Res.* **13**, 716 (1953).
22. M. W. Woods, J. C. Hunter and D. Burk, *Proc. Amer. Ass. Cancer Res.* **3**, 163 (1960).
23. M. Woods, D. Burk and J. Hunter, *Proc. Amer. Ass. Cancer Res.* **3**, 74 (1959).
24. O. Lindberg and L. Ernster, *Protoplasmatologia*, Band III, A4. Springer-Verlag, Vienna (1954).
25. L. Pasteur, *Studies on Fermentation* (F. Faulkner and D. C. Robb, Tr.). Macmillan, London (1879).
26. K. C. Dixon, *Biol. Rev.* **12**, 431 (1937).
27. D. Burk, in *Some Fundamental Aspects of the Cancer Problem* (Occasional Publications of the A.A.A.S. no. 4). Science Press, New York (1937).
28. D. Burk, in *Cold Spring Harbor Symposia on Quantitative Biology*, VII, p. 421. Darwin Press, New Bedford, Mass. (1939).