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RELATIONSHIP OF BIOCHEMICAL DRUG EFFECTS TO THEIR ANTITUMOR ACTIVITY—II

DIACRIDINES AND MEMBRANE-RELATED REACTIONS

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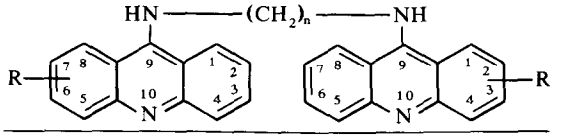
Abstract—A method is presented that determines the degree of attachment of cancer cells to normal cells. This method may be useful in determining the extent to which treatment of normal cells (or of a tumor-bearing host) with a particular chemotherapeutic agent may affect the degree of attachment of cancer cells to the normal cells. The effects of several diacridines upon this process are described. In addition, we have determined the ability of individual diacridines to alter the permeability of P-388 cells; this effect has been related to their antitumor properties. In general, the most effective antitumor diacridines are those that cause minimal disruption of cell permeability. Conversely, diacridines that disrupt cell permeability tend to have poor antitumor properties. It is considered that the toxicity of these compounds may be a necessary consequence of the assays used for testing anticancer agents, and may not necessarily be related to their antitumor activity.

We initiated a series of studies to distinguish the essential antitumor activities of chemotherapeutic agents from unnecessary toxic effects that such agents may have. We used diacridines as model compounds in these studies because of our experience with these compounds over a period of years.

We had shown previously that, although most diacridines that we synthesized as antitumor agents [1] are excellent bifunctional intercalators of nucleic acids [2, 3], and although they specifically inhibit reactions such as the initiation of RNA synthesis [4] or the processing of 45S RAN [5], their antitumor activities do not correlate with their abilities to inhibit such nucleic acid-related reactions. Among the known biological activities of the diacridines, we found that a significant correlation exists only between their antitumor activity and their effect on cell agglutination [6]. From these and other [7] experimental findings, it was hypothesized that the antitumor effects of diacridines may be mediated through their actions on cell membranes [6, 7].

We have now studied the effects of a number of diacridines (Table 1) on a variety of membrane-related reactions and related them to their antitumor activity. We have also developed an *in vitro* assay that determines the degree of aggregation of cancer cells to normal cells and the changes occasioned by chemotherapeutic or other agents upon this aggregation. Such an assay may provide a useful *in vitro* measure of the tendency of individual cancer cells to aggregate within normal tissues. It may also be useful in identifying compounds that affect those aspects of

Table 1. Structures of diacridines evaluated in these studies*



Drug code number	CH_2 groups N	R
1	2	
2	4	
3	5	
4	6	
5	6	4-Ethyl-
6	6	3-Nitro-
7	6	2-Methoxy
8	6	3-Propoxy
9	6	4-Methoxy
10	6	4-Butoxy
11	7	
12	8	
13	8	4-Ethyl
14	8	2-Methoxy
15	8	3-Methoxy
16	8	4-Methoxy
17	8	3,5-Dimethoxy
18	8	3-Methoxy-7-chloro
19	8	4-Ethoxy
20	8	3-Propoxy
21	8	3-Nitro
22	8	3-Chloro
23	8	3-Bromo
24	8	4-Methylthio
25	8	4-Butoxy
26	10	
27	12	
28	12	4-Aza

* The drug code numbers correspond to those in the text.

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metastasis [8] which rely upon the degree of association of cancer cells with normal cells.

The results indicate that the diacridines, which have very specific and prominent cellular toxicities related to nucleic acid synthesis and processing, also have membrane-related effects that may be important in the expression of their antitumor activities.

MATERIALS AND METHODS

Materials. P-388 and L-1210 cells were obtained from the Arthur D. Little Co., Boston, MA. The L-5178Y, Sarcoma 180 and Walker 256 cells were obtained from the frozen cell stocks of this department. The 3T3 and the CHO cells were obtained from the American Type Tissue Culture Collection, Camden, NJ. The media, sera, trypsin-EDTA and penicillin + streptomycin were obtained from Gibco, Grand Island, NY. Costar dishes and 25-ml and 75-ml polystyrene flasks were obtained from Corning Glassworks, Corning, NY. [5,6-^{3}H]Uridine (50 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA. All reagents used were analytical grade.

Growth and maintenance of cells. The P-388, L-1210 and L-5178Y cells were maintained in Fischer's medium with 10% horse serum, as described previously [4]. All other cell lines were maintained in DMEM (Dulbecco's modification of Eagle's minimum essential medium) containing 100 units/ml penicillin and 100 μg/ml streptomycin plus 10% fetal bovine serum [3]. All cell lines were grown in a Hotpack incubator at 37° (95% humidity, 5% CO_2).

Measurement of permeability of the P-388 cells. Logarithmically growing P-388 cells (1–3 × 10^5 cells/ml), in Fischer's medium containing 10% (v/v) horse serum [7], were labeled with [^{3}H]uridine (10 μCi/ml), at 37°, for 30 min. The cells were then pelleted, washed twice with a similar volume of fresh growth medium plus 10% horse serum at 37°, and then incubated at 37° for 1 hr in a similar volume of fresh medium containing 10% horse serum plus 50 μg/ml of non-radioactive uridine. The cells were again washed as above and resuspended in a similar volume of growth medium containing 10% horse serum plus 25 μg/ml of nonradioactive uridine. The cells were exposed to 10^{-5} M diacridine, incubated for 20 min at 37°, and centrifuged at 3000 rpm for 10 min at 4°; 0.2-ml samples of the supernatant fluid were assayed for radioactivity in triplicate.

The equation $\Delta P = (T_{20} - C_0)/(C_{20} - C_0)$ was used to calculate the relative permeability change P, induced by the diacridines. The values of T_{20} and C_{20} are cpm in the incubation medium after 20 min of incubation in the presence and absence of the diacridine respectively. The value C_0 is cpm in the incubation medium immediately prior to the addition of the diacridine.

Determination of the attachment of tumor cells to confluent CHO or 3T3 cells. The P-388 cells were labeled with [^{3}H]uridine and washed as described above for the permeability measurements. The radioactive P-388 cells (5–6 × 10^5) were suspended in 3 ml of growth medium containing 25 μg uridine/ml and added to 1.9 × 10^6 confluent CHO or 3T3 cells/well from which the growth medium had been removed.

In some experiments, the monolayers of CHO or 3T3 cells were pretreated with diacridines by incubating the confluent monolayer of CHO cells in complete growth medium with 10^{-5} M diacridine for 15 min at 37°. The cells were then quickly washed twice with fresh growth medium, and the attachment assay was initiated immediately, as follows.

The cancer cells were allowed to attach, for 1 hr at 37°, to the CHO or 3T3 cells with occasional gentle rotation of the 6-well Costar dishes. Unattached cancer cells were removed by aspiration, and the monolayer was washed three times with 3 ml of fresh growth medium. The residual cells were trypsinized, suspended in fresh medium, and centrifuged (1000 *g*, 6 min). The medium was discarded, and the cells were treated with 1.5 ml of 10% trichloroacetic acid (TCA) at 4°. The TCA-insoluble material was collected by centrifugation and further washed with 10% TCA. The radioactivity in the precipitate and in portions of the supernatant fraction was then determined by counting the samples after addition of 10 ml Aquasol. A correction for the spurious uptake of radioactivity from the cell medium into the CHO cells was made, though this was generally insignificant.

Determination of the concentrations of diacridines bound to plasma membranes. Membranes of normal human red blood cells were isolated [9] from recently outdated blood transfusion center supplies and suspended in phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, in 0.15 M NaCl) at 300 μg protein/ml. Diacridines were added to a final concentration of 10^{-5} M and incubated at 37° for 15 min. This period of time was found to be adequate for maximal uptake of diacridines by the membranes. The membranes were then collected by centrifugation (15,000 *g*, 10 min), washed several times with phosphate-buffered saline and resuspended in the original volume of phosphate-buffered saline. The membrane suspension was made 1% in sodium dodecyl sulfate and dissolved, and the fluorescence was determined at an excitation wavelength of 400 nm and an emission wavelength of 490 nm. The fluorescence values of untreated membrane controls dissolved in 1% sodium dodecyl sulfate and of reagent blanks in buffer served as experimental fluorescence blank values. The calibration curve was made by determining, similarly, the fluorescence of various concentrations of diacridines in the same medium as above.

Additional assays. Protein was measured by the method of Lowry *et al.* [10] with bovine serum albumin (Fraction III) as standard. The cells were routinely tested for mycoplasma contamination [11] and were found to be negative.

RESULTS

Attachment of cancer cells to quiescent normal cells. Incubation of P-388 cells with monolayers of quiescent CHO cells resulted in the attachment of P-388 cells to the CHO cells (Fig. 1). At the ascending linear portion of the curve, approximately 3% of the P-388 cells attached to the CHO cells. A

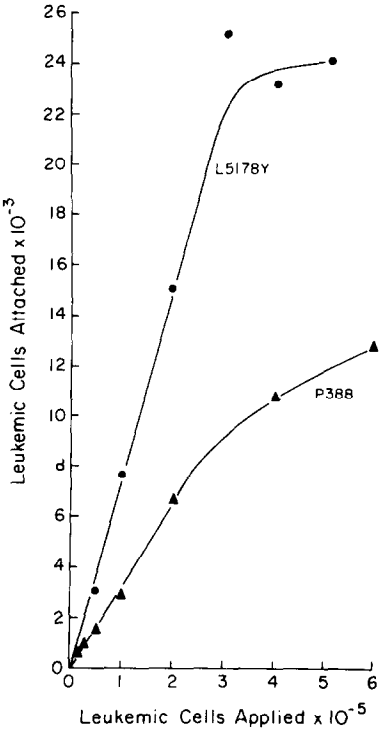

Fig. 1. Attachment of P-388 and L-5178Y cells to confluent CHO cells. Logarithmically growing leukemia cells were allowed to attach to monolayers of 1.9×10^6 confluent CHO cells, and the number of attached cells was determined (see Materials and Methods). Similar results were obtained when confluent 3T3 cells were used in place of the CHO cells.

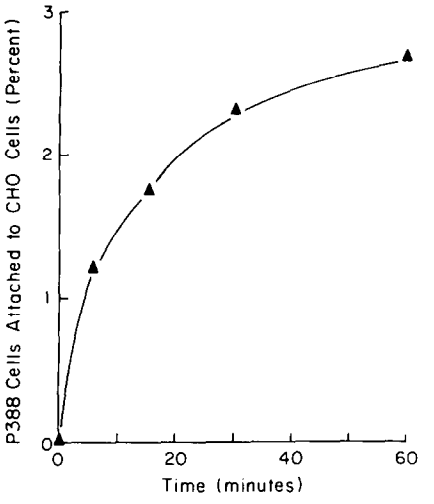

Fig. 2. Time dependence of the attachment of P-388 cells to monolayers of confluent CHO cells. This experiment was performed as described in the legend to Fig. 1 and in Materials and Methods, except that a constant number of P-388 cells was used (5×10^5 cells) and the time of incubation was varied.

qualitatively similar result was obtained when L-5178Y cells were used in place of P-388 cells, except that approximately 8% of the added L-5178Y cells attached to the CHO cells.

The attachment was time dependent. As shown in Fig. 2, the attachment of P-388 cells reached plateau levels at about 60 min. We found this result to be similar to that obtained with other cancer cell lines, and we, therefore, used this period of exposure of cancer cells to the CHO cells in the subsequent related experiments.

We observed the fundamental phenomenon of attachment of cancer cells to normal cells to be qualitatively reproducible with the various cancer cells that we studied and with monolayers of either CHO or 3T3 cells. It appeared that the degree of attachment varied with the state of growth of the CHO or 3T3 cells; however, we did not pursue this aspect of the phenomenon any further and preferred to standardize the incubation conditions by using confluent cell monolayers.

Effects of various diacridines on the attachment of cancer cells to CHO cells. In these experiments, we pretreated the CHO cells with diacridines and determined the effect of this pretreatment upon the degree of attachment of cancer cells to the CHO cells. This experiment was performed as an *in vitro* counterpart of a portion of the metastatic process, to evaluate whether drug treatment of the host can alter the association of cancer cells to normal cells.

We kept at a minimum (15 min) the exposure of the CHO cells to the diacridines and performed the complete experiment within approximately 90 min. As indicated in Materials and Methods, the association of the diacridines with plasma membranes was maximized in 15 min. It was anticipated that, in this time period, any consequent physical chemical effect

Table 2. Effect of pretreatment of CHO cells with diacridine No. 16 (4-methoxy C-8) on the attachment of various cancer cells to CHO cells*

Treatment of CHO cells	Cancer cells: P-388 (number of cancer cells attached to CHO cells)	Sarcoma 180	Walker 256
None	5,400	27,200	21,200
Exposed to diacridine No. 16	7,238	40,900	86,800

* Monolayers of confluent CHO cells (approx. 1.9×10^6 cells) were treated with 10^{-5} M diacridine for 15 min, washed, and exposed to approximately 5×10^5 radioactive cancer cells as described in Materials and Methods. The averages of three experimental values are given. The standard deviations were less than ± 10%.

that the plasma membrane bound diacridines had upon the attachment of cancer cells to normal cells was expressed. This short exposure also minimized any secondary effects the diacridines may have upon the plasma membrane through structural changes of the plasma membrane that result from inhibition of nucleic acid metabolism [1].

Using these conditions (Table 2), we found that pretreatment of the normal CHO cells with one of the effective antitumor diacridines, 4-methoxy C-8 (No. 16, see Table 1) caused variable increases in the attachment of the P-388 leukemia cells and the Sarcoma 180 and Walker 256 cancer cells to the diacridine-treated CHO cells. This effect varied from a 30% increase to a 300% increase and was reminiscent of the variable effects that a chemotherapeutic agent may have upon tumors of different origin.

The converse experiment, in which the effect of the treatment of CHO cells by various diacridines upon the attachment of one cancer cell line, the L-5178Y leukemia cells, to the CHO cells was also studied. An experiment similar to the above was performed in which the CHO cells were pretreated for 15 min with diacridines that differed in their antitumor activity (defined by %ILS = percent increase in life span of the treated animals over the life span of the controls) (Table 3). Diacridines No. 9 (4-methoxy C-6) (%ILS = 60), No. 10 (4-butoxy C-6) (%ILS = 20) and No. 16 (4-methoxy C-8) (% ILS = 90) enhanced the attachment of L-5178Y cells to the CHO cells, while a similar pretreatment of the CHO cells with diacridine No. 25 (4-butoxy C-8) (%ILS = 20) decreased the attachment of the L-5178Y cells to the diacridine-treated CHO cells.

These results indicated that even circulating blood cells, such as the leukemia P-388 and the L-5178Y cells, could attach to normal host cells. This result is in agreement with the earlier finding that leukemia cells such as the L-1210 cells, when administered intraperitoneally into mice, can become embedded and grow within normal tissues [12]. They also indicated that short-term exposure of normal cells to diacridines altered membrane-related interactions between cancer cells and normal cells, resulting in differential degrees of adhesion between these cells. These effects varied with the diacridine used and with the cancer cells used and reinforced our earlier findings on the variable effects diacridine exhibited upon the self-agglutination of cancer cells [7].

Table 3. Effect of pretreatment of CHO cells with various diacridines on the attachment of L-5178Y cells to the CHO cells*

Diacridine pretreatment Compound No.	%ILS	Change in attachment of L-5178Y cells (%)
9	60	+230
10	20	+250
16	90	+140
25	20	−30

* Monolayers of confluent CHO cells (approx. 1.9×10^6 cells) were treated with 10^{-5} M diacridine for 15 min, washed, and incubated with approximately 5×10^5 radioactive L-5178Y cells as described in Materials and Methods. Control values: 30,000 L-5178Y cells were attached to the CHO cells. The averages of three experimental values are given. The standard deviations were less than ± 10%.

Effect of diacridines on cellular permeability. We measured the basal permeability of the cell plasma membrane to the intracellular nucleotides, and its alteration in the presence of various diacridines by using P-388 cells, prelabeled with [^{3}H]uridine (Materials and Methods). Individual diacridines differentially affected the release of acid-soluble nucleotides from P-388 cells (Fig. 3). These permeability effects of the individual diacridines varied from causing an inhibition of extrusion of acid-soluble radioactive nucleotides (see compounds to the left of dashed line) to causing an extensive liberation

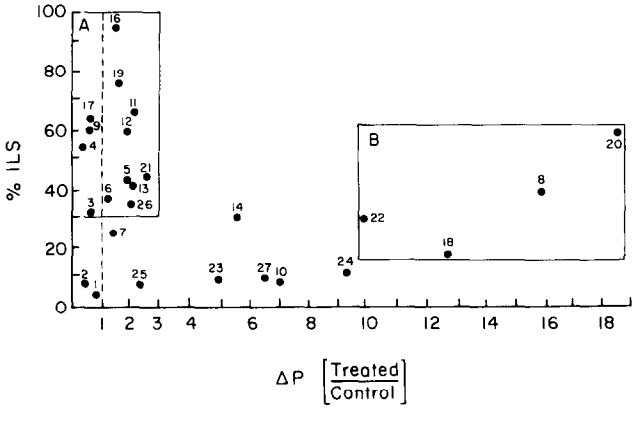

Fig. 3. Relationship between the change in permeability induced by exposure of P-388 cells to diacridines and the antitumor effectiveness of the diacridines. The change in permeability is determined as described in Materials and Methods. The %ILS (percent increase in life span of P-388 bearing mice) following treatment with the various diacridines is taken from an earlier publication [8]. The numbers refer to the code numbers of the diacridines given in Table 1.

of acid-soluble radioactive nucleotides into the medium. When the effect of each diacridine on the permeability of P-388 cells was plotted against the effect of the individual diacridines on the percent increase of the life span of P-388 tumor bearing mice (%ILS), it appeared that the most effective antitumor diacridines did not cause large permeability changes on the P-388 cells. As could be anticipated, the converse was not necessarily true; some diacridines that did not greatly affect the cellular permeability, were not effective antitumor agents.

In Box A (Fig. 3), we enclosed the diacridines that increased the life span of mice bearing P-388 tumors by more than 30%. Most of the diacridines in this category had minimal effects on the permeability of these cells and did not enhance their basal permeability by more than 2- to 3-fold. This did not appear to constitute a great change in cellular permeability because the total amount of acid-soluble radioactivity released from the cells by these diacridines accounted only for 10–20% of the total intracellular acid-soluble pool of uridine-labeled compounds, as compared to 5–10% for the control cells.

On the other hand, those diacridines that enhanced the permeability of the P-388 cells by more than three times the control rate were largely inactive as antitumor agents. These included diacridines 23, 14, 27, 10 and 24.

The diacridines in Box B have two common characteristics. They all caused extensive increases in the permeability of the P-388 cells, and all carry lipophilic substituents in position 3 of the acridine rings. Diacridine No. 20, 3-propoxy C-8 diacridine, was most interesting in this regard, because it was the only diacridine with considerable antitumor activity which caused very extensive changes in cell permeability.

The degree of electronegativity of the substituents in position 3 of the acridine rings also seemed to correlate with the increase in the permeability of the P-388 cells. This characteristic is apparent in diacridines carrying chloro- (No. 22), bromo- (No. 23) and nitro- (No. 6 and No. 21) groups in position 3 of the acridine rings.

In all of these experiments the radioactivity released into the medium consisted of uridine and of uridine nucleotides, primarily di- and tri-phosphates in most cases [6]. Only diacridines No. 10 and No. 25 (see Table 1), which caused large permeability changes (Fig. 3), also release appreciable amounts of high molecular weight nucleic acids [6].

Binding of diacridines to plasma membranes. We also determined the ability of those diacridines that cause minimal permeability changes (up to 2.5-fold over basal levels) to bind to plasma membranes. To avoid any possible contaminations with other cell fractions which could contain nucleic acids, such as the endoplasmic reticulum, we measured the degree of association of various diacridines with red blood cell plasma membranes as a model of nucleic acid-free, normal plasma membranes.

Within this group of diacridines, we found that the binding to the plasma membrane could vary by as much as 20-fold (Fig. 4). Diacridines 1, 2 and 3 were

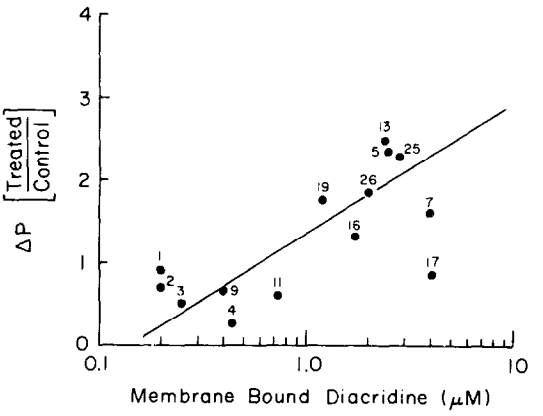

Fig. 4. Relationship between the binding of diacridines to RBC membranes and the change in permeability induced by exposure of P-388 cells to diacridines. The binding of the diacridines to the RBC membranes was determined as described in Materials and Methods. The numbers refer to the code numbers of the diacridines given in Table 1.

bound minimally by these plasma membranes, and tended to *decrease* the basal cellular permeability. It is interesting to note that, among all the diacridines that we have synthesized, the latter were the only diacridines that were functionally mono-intercalators of DNA because of the short chain connecting the two acridine rings [13].

At the other extreme, the diacridines that were more heavily concentrated by the plasma membranes caused greater permeability changes and were effective antitumor agents. Within this group of diacridines that cause minimal permeability changes, the most effective antitumor diacridines (No. 19, No. 16 and No. 17) were bound relatively extensively by the membranes.

DISCUSSION

The diacridines were synthesized to create better intercalators of DNA that would kill tumor cells more efficiently than the parent compounds [1]. These original expectations have been fulfilled [1–3]. However, the accumulated evidence from the present and earlier studies [2, 5] indicates that the diacridines associate with cell membranes and affect various reactions related to the plasma membrane. These include cell agglutination [2, 5], attachment of cancer cells to normal cells, and cell permeability.

It is our contention that the assays currently in use for the identification of anticancer compounds necessarily select for toxic compounds. These assays select compounds that prevent or delay the death of animals to which large numbers of tumor cells have been administered.

However, we should consider that, after administration of cancer cells to animals, two cancer cell compartments are formed: a large compartment containing the bulk of the administered cancer cells and a smaller compartment containing individual cancer cells sequestered within normal tissues. Such animal assays necessarily select compounds that are toxic enough to destroy the large bulk of the administered

cancer cells. We do not know, however, whether this toxicity is also necessary to prevent the outgrowth of the few cancer cells that are sequestered within normal host tissues.

Experimental evidence indicates that toxic compounds or tissue trauma may promote the outgrowth of individual cancer cells within normal host tissue. For instance, trauma in the form of manipulation of the liver lobes promotes the outgrowth of intraportally administered cancer cells [14]. Similarly, induction of growth of administered cancer cells occurs if the animals are subjected to X-ray treatment [15] or to thoracic lymph drainage [15]. Furthermore, following partial hepatectomy or CCl_4 treatment, the expression of the *src*, the *ras* and the *onc* genes can be detected in the regenerating cells [16].

We have attempted to distinguish the desirable antitumor effects of the diacridines from their undesirable toxic effects. For this reason, we have related their various biochemical effects to their %ILS (percent increase in life span of tumor-bearing animals), in order to determine which biochemical effects correlate best with %ILS. Both the present results and the earlier results [2, 5] indicate that the membrane-related effects of the diacridines correlate better with the %ILS than their effect on nucleic acid synthesis [2, 5]. The question that we shall have to seriously consider is whether the toxicity that is inherent in the anticancer compounds that are currently in use may promote the outgrowth of individual cancer cells isolated within normal host tissue. It is for this reason also that we shall have to develop specific assays that distinguish between desirable antitumor effects from what may prove to be undesirable toxic effects that promote, rather than prevent, the outgrowth of individual cancer cells sequestered within normal tissues of the host.

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REFERENCES

1. E. S. Canellakis, Y. H. Shaw, W. E. Hanners and R. A. Schwartz, *Biochim. biophys. Acta* **418**, 277 (1976).
2. T. K. Chen, R. Fico and E. S. Canellakis, *J. med. Chem.* **21**, 868 (1978).
3. A. H. Sarris, C. Niles and E. S. Canellakis, *Biochim. biophys. Acta* **474**, 268 (1977).
4. R. M. Fico and E. S. Canellakis, *Biochem. Pharmac.* **26**, 275 (1977).
5. R. M. Fico, T. K. Chen and E. S. Canellakis, *Science* **198**, 53 (1977).
6. E. S. Canellakis and T. K. Chen, *Biochem. Pharmac.* **28**, 1971 (1979).
7. E. S. Canellakis and R. A. Bellantone, *Biochim. biophys. Acta* **418**, 290 (1976).
8. I. J. Fidler, D. M. Gersten and I. R. Hart, in *Advances in Cancer Research* (Eds. G. Klein and S. Weinhouse), Vol. 28, p. 150. Academic Press, New York (1978).
9. T. L. Steck and J. A. Kant, *Meth. Enzym.* **31**, 172 (1974).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. L. Hayflick, in *Tissue Culture Methods and Applications* (Eds. P. F. Kruse, Jr. and M. K. Patterson, Jr.), p. 722. Academic Press, New York (1973).
12. H. E. Skipper, F. M. Schabel and W. S. Wilcox, *Cancer Chemother. Rep.* **31**, 3 (1964).
13. L. P. G. Wakelin, M. Romanos, T. K. Chen, D. Glaubinger, E. S. Canellakis and M. J. Waring, *Biochemistry* **17**, 5057 (1978).
14. B. Fisher and E. R. Fisher, *Science* **130**, 918 (1959).
15. S. A. Eccles and P. Alexander, *Nature, Lond.* **257**, 52 (1975).
16. M. Goyette, C. J. Petropoulos, P. R. Shank and N. Fausto, *Science* **219**, 511 (1983).