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EFFECTS OF HEXACHLOROCYCLOHEXANE ISOMERS ON THE MITOGENIC RESPONSE OF BOVINE LYMPHOCYTES

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Abstract—The α-, β-, γ- and δ-isomers of hexachlorocyclohexane (HCH) were evaluated as modifiers of the mitogenic response in cultured bovine lymphocytes. None of the four isomers were mitogenic alone; however, the concurrent administration of αHCH and phytohemagglutinin-P (PHA) stimulated DNA synthesis in lymphocyte cultures 2.2-fold over that obtained with PHA alone. βHCH was inactive in the test system whereas both γ- and δHCH severely inhibited the PHA response. Treatment of lymphocytes with the microsomal enzyme inhibitor SKF-525A blocked the co-mitogenic activity of αHCH without affecting the basal PHA response. The role of hexachlorocyclohexane-sensitive metabolic systems in the regulation of mitogenesis in lymphocytes is discussed.

The treatment *in vitro* of small lymphocytes with phytohemagglutinin-P (PHA) or other mitogenic agents provides excellent systems for studying the sequence of molecular events associated with the transition of a cell from a restricted to an active growth state. In this system, the administration of PHA is attended by an immediate acceleration of phosphatidyl inositol turnover which leads, in turn, to an acceleration of RNA and protein synthesis, the triggering of DNA and chromatin replication, and the activation of mitotic processes leading to cell division [1–4]. The acceleration of phosphatidyl inositol turnover appears to be a critical step in the induction of growth by the lectin, since lindane (γHCH), a hexachlorocyclohexane cogener of inositol and inhibitor of CDP-diglyceride-inositol transferase [5], blocks both the initial acceleration of phosphatidyl inositol turnover and the subsequent induction of RNA, protein, and DNA synthesis in human lymphocytes [6]. These results suggest that inositol metabolism may play a central role in the control of small lymphocyte metabolism. To explore this area further, we have tested other isomers of HCH for effects on the mitogenic process. The delta-isomer, like γHCH, was strikingly inhibitory to PHA-treated lymphocytes, whereas the beta-isomer was inactive. The alpha-isomer proved to be distinctive in this system. When administered along with PHA, it acted synergistically to promote DNA synthesis in certain bovine lymphocytes. This co-mitogenic action was prevented by SKF-525A, an inhibitor of the membrane-associated cytochrome P-450 systems which are involved in the metabolism of polychlorinated cyclo-hydrocarbons. When administered separately, αHCH, in addition to the above action, exhibited a unique capacity for preventing the delayed DNA synthesis associated with the mixed lymphocyte response in these cultures. These results are discussed with respect to possible roles of membrane metabolism in the control of genetic expression of small lymphocytes responding to mitogenic stimuli.

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MATERIALS AND METHODS

Lymphocytes were isolated from retropharyngeal lymph nodes of freshly slaughtered adult cattle (Oscar Mayer Co.). Isolation procedures were carried out at room temperature under sterile conditions in a laminar-flow hood. After dissecting away fat and connective tissue, the intact lymph nodes were placed in 70% ethanol for 5 min to fix *in situ* any peripheral bacterial contamination. Lymph nodes were then rinsed three times in a modified Eagle's medium containing 10% bovine serum (BEHM [7]) and gentamicin (50 μg/ml) and finely minced with scissors. The minced tissue, suspended in the media, was filtered through two layers of a fine-mesh cheesecloth to separate connective tissue and other debris from the dispersed lymphocytes. The latter were collected by centrifugation (150 *g* for 15 min) and washed three times by alternate suspension in, and centrifugation from fresh medium. Care was taken to separate the lymphocytes from the red blood cells which form the bottom pellet. Lymphocytes were finally suspended at a concentration of 5–6 × 10^6 cells/ml in BEHM containing gentamicin and incubated at 37° overnight in a spinner bottle with gentle stirring and a 5% CO_2–95% air atmosphere. The following day the lymphocyte concentration was diluted to 2–3 × 10^6 cells/ml and 5-ml aliquots of the cell suspension were transferred to 10-ml conical centrifuge tubes and placed on a 4° slant in a 37° incubator.

Phytohemagglutinin-P, obtained as a vial of sterile powder from Difco Laboratories (Detroit, MI) was diluted to 5 ml with sterile water. PHA was added to cultures at a final dilution of 1:1000 of this stock solution at the time the lymphocytes were planted. This point constitutes time 0 in all experiments. αHCH was purchased from Aldrich Chemical Co. (Milwaukee, WI) and the β-, γ- and δ-isomers were obtained from Professor Paul Lichtenstein, Entomology Dept., University of Wisconsin. The hexachlorocyclohexanes were added at the intervals and concentrations indicated in the individual experiments. Dimethyl sulfoxide (DMSO) (spectrophotometric

grade, Aldrich Chemical Co.) was used as the vehicle for the hexachlorocyclohexanes, their concentrations adjusted such that the final DMSO concentration was always 0.5%.

Growth response of the bovine lymphocytes was quantified by measuring the incorporation of [^{3}H]thymidine. Cultures remained undisturbed until 0.25 ml of [^{3}H]thymidine (8 μCi/ml, 6.7 Ci/m-mole, New England Nuclear, Boston, MA was added to each tube. Tubes were shaken gently to distribute the radioactivity and returned to the incubator for 2 hr. The incubation was stopped by chilling the tubes in an ice-water slurry, adding 5 ml of cold saline and centrifuging at 1000 *g* for 3 min. The supernatant was decanted and the cells resuspended in a few drops of saline and frozen at −20°. For analysis the stored cells were quickly thawed, resuspended, and precipitated with 5 ml of ice-cold 4% perchloric acid ($HClO_4$). The precipitate was washed sequentially with 4% $HClO_4$, 80% ethanol, 100% ethanol, and 4% $HClO_4$ and finally resuspended in 1 ml of 6% $HClO_4$ and heated at 80° for 1 hr. Radioactive incorporation was measured by mixing 0.5-ml aliquots of the hot acid-soluble material with 10 ml Scintosol (Isolab) and counting in a Searle Isocap liquid scintillation spectrometer with a 45 per cent counting efficiency.

RESULTS

Enhancement of PHA-mediated transformation of bovine lymphocytes by αHCH. As has been described previously by this laboratory [8, 9], the response of cultured bovine lymphocytes to phytohemagglutinin involves the characteristic differentiation into blast cells and induction of cell replication. During the first 24 hr after phytohemagglutinin addition, the incorporation of thymidine into DNA remains at a low level. Thereafter, the ability of stimulated lymphocytes to incorporate thymidine increases rapidly to reach a peak at 40–50 hr. Thymidine incorporation then drops to low levels between 80 to 100 hr reflecting the synchrony of the response. Control cultures without phytohemagglutinin are essentially unable to incorporate thymidine up to 80 hr; however, after this time lymphocytes in these experiments engage variably in a proliferative response and exhibit increased thymidine uptake. This delayed response has been shown to be due to a mixed lymphocyte reaction and can be avoided if cells are derived from the lymph nodes of a single animal. These responses are illustrated in Fig. 1. The concurrent addition of a non-toxic dose (0.01 mM) of αHCH with PHA greatly enhanced the ability of lymphocytes to incorporate thymidine over cultures of lymphocytes treated with PHA alone. Measuring thymidine incorporation in a series of seven experiments at 48–50 hr, the enhancement by αHCH was 2.2-fold (range 1.6 to 3.2x) over PHA stimulation alone. This same dose of αHCH in the absence of PHA failed to stimulate thymidine incorporation at any time-point, thereby indicating that αHCH acts as a co-mitogen rather than as a primary mitogen. An interesting aspect of the cultures treated with αHCH alone was a lack of the delayed thymidine incorporation; it appears that αHCH completely suppressed the mixed lymphocyte reaction which is observed in control cultures beyond 80 hr. The decline in the number of viable cells throughout the course of the experiment, as monitored by Trypan Blue exclusion, was nearly equivalent between αHCH-treated and control cultures, suggesting that these effects of αHCH at a low dosage were not a consequence of general cell toxicity.

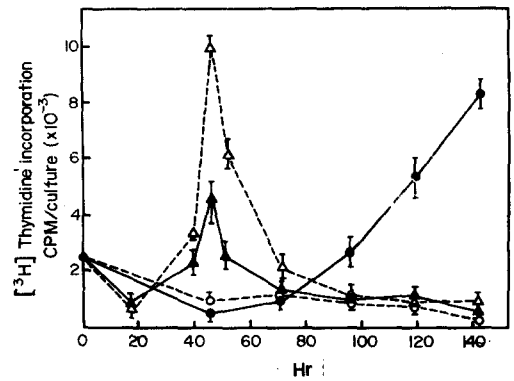

Fig. 1. Time course of [^{3}H]thymidine incorporation by lymphocytes after mitogen treatment. Five-ml cultures were pulsed for 2 hr with [^{3}H]thymidine at the indicated times after the start of cultures. PHA and/or αHCH were added to cultures at time zero. Key: (●——●) control, (○----○) αHCH (0.01 mM), (▲——▲) PHA and (△--△) PHA + αHCH (0.01 mM). Points are the means of triplicate determinations ± S. E.

Influence of treatment schedule on the enhancement of the PHA response by αHCH. A pair of experiments were designed to delineate possible critical windows in the enhancement of PHA-stimulated DNA synthesis in lymphocytes by αHCH. In the first experiment, cultures were planted in the presence of PHA and 0.01 mM αHCH was added at intervals from 0 to 46 hr after PHA addition. [^{3}H]thymidine incorporation was measured in all cultures in the interval from 48 to 50 hr. As shown in Fig. 2, αHCH had to be

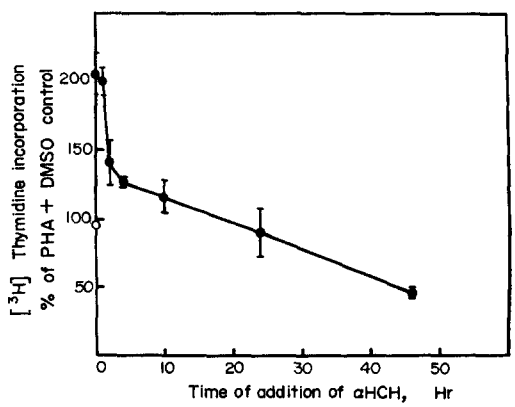

Fig. 2. Effect of varying the time of addition of αHCH on the mitogenic response of PHA-treated lymphocytes. αHCH dissolved in DMSO (final concentrations 0.01 mM and 0.5%, respectively) was added to cultures at the indicated times after planting cultures in the presence of PHA. Cultures were then pulsed with [^{3}H]thymidine from 48 to 50 hr. Incorporation of [^{3}H]thymidine is expressed as per cent of values obtained for PHA + DMSO cultures. Key: (○) PHA without DMSO added and (●——●) PHA + αHCH.

added to the cultures nearly concurrently with the lectin to obtain maximal enhancement. Addition of αHCH later than 10 hr after PHA yielded no enhancement and indeed αHCH addition at 46 hr was inhibitory (45 per cent of the PHA–DMSO control level) to [^{3}H]thymidine incorporation. Varying the time of addition of the solvent, DMSO, to PHA-stimulated cultures had no effects on [^{3}H]thymidine incorporation.

Since this experiment suggested dual effects of αHCH, co-mitogenic as well as inhibitory ones, a second type of experiment was performed to attempt to separate the components of the response. PHA and αHCH were added to cultures at 0 hr. At the indicated times the lymphocytes were pelleted and resuspended in BEHM containing PHA without αHCH. The results of this type of protocol (Fig. 3) show that exposure of PHA-stimulated lymphocytes to concurrently administered αHCH, regardless of the length of exposure time, always produced enhanced [^{3}H]thymidine incorporation. However, the shorter the exposure of αHCH, the greater was the enhancement. A 1-hr pulse of αHCH promoted a 3-fold increase in [^{3}H]thymidine incorporation, whereas a 48-hr exposure of cultures to αHCH produced only a 1.8-fold enhancement. Again, both aspects of the response to αHCH are manifested, suggesting the possibility that αHCH exerts a co-mitogenic effect on one subpopulation of lymphocytes and exerts an inhibitory effect on another.

Effect of SKF-525A and αHCH on DNA synthesis. SKF-525A is an agent known to inhibit the microsomal drug-metabolizing pathways. To assess whether metabolic activation is required before αHCH can exert its co-mitogenic effects, lymphocytes were cultured in the presence of combinations of PHA, αHCH and SKF-525A. As shown in Fig. 4, the level of DNA synthesis measured at 48–50 hr was stimulated 2.6-fold by addition of αHCH to PHA-treated cultures. Concurrent addition of 10 μM SKF-525A to cultures stimulated with PHA and αHCH prevented

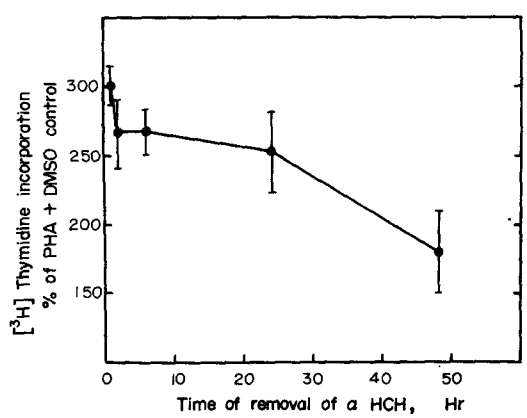

Fig. 3. Effect of transferring PHA + αHCH-stimulated lymphocytes to αHCH-free media on the mitogenic response. PHA and 0.01 mM αHCH were added to cultures at time zero. At the indicated times, the lymphocytes were centrifuged at 150 *g* for 15 min and resuspended in fresh media containing PHA alone. Cultures were then pulsed with [^{3}H]thymidine from 48 to 50 hr. The data are expressed as per cent of the PHA + DMSO control.

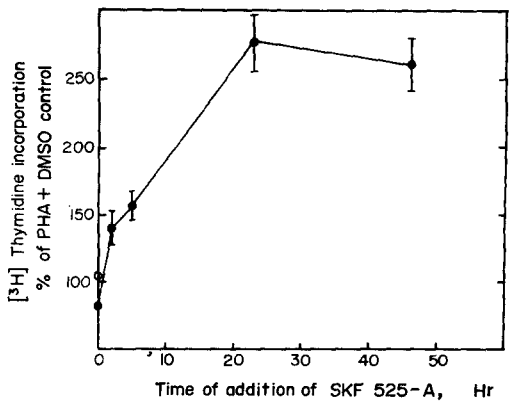

Fig. 4. Effect of varying the time of addition of SKF-525A on the co-mitogenic action of PHA and αHCH on lymphocytes. PHA and 0.01 mM αHCH were added to cultures at zero hour. At the indicated times, 10 μM SKF-525A (dissolved in saline) was added to the cultures. Cultures were pulsed with [^{3}H]thymidine from 48 to 50 hr. Incorporation of [^{3}H]thymidine is expressed as per cent of values obtained for PHA + DMSO. Addition of SKF-525A to PHA + DMSO cultures was without effect (○). The closed symbols (●——●) indicate PHA + αHCH + SKF-525A.

the enhancing effect of αHCH. When SKF-525A was added 24 hr or later after αHCH, the drug did not prevent the enhancement of [^{3}H]thymidine incorporation by αHCH. Addition of SKF-525A alone had no apparent effect on the PHA-induced mitogenic response. These results suggest that it might be necessary to metabolize αHCH in order to have it act as a co-mitogen.

Comparison of hexachlorocyclohexane isomers for their effects on the mitogenic response of lymphocytes. Four isomers (α, β, γ and δ) of hexachlorocyclohexane were evaluated for effects on DNA synthesis in PHA-stimulated lymphocytes over a 20-fold range of concentrations. The hexachlorocyclohexane isomers and PHA were both added at 0 hr and the cultures pulsed with [^{3}H]thymidine during the interval from 48 to 50 hr. The results are shown in Fig. 5. αHCH was increasingly stimulatory over a 5–50 μM range. Beyond these concentrations αHCH became toxic to the cells and the solubility of the compound also became limiting. βHCH was without effect at all doses tested except the highest (100 μM) where inhibition was 42 per cent. Both the γ- and δ-isomers were toxic at levels greater than 10 μM. Thus, the α hexachlorocyclohexane isomer was the only one to show stimulatory effects on PHA-mediated DNA synthesis. None of the four isomers, when tested at a concentration of 0.01 mM in the absence of PHA, exerted any mitogenic activity.

DISCUSSION

The small lymphocyte is a highly repressed cell with little cytoplasm, a low metabolic activity, and is arrested in G_0. This restricted state is rapidly overcome by treatment with mitogenic agents, like PHA, Con A or poke weed mitogen. These agents combine initially with components of the cell surface to acti-

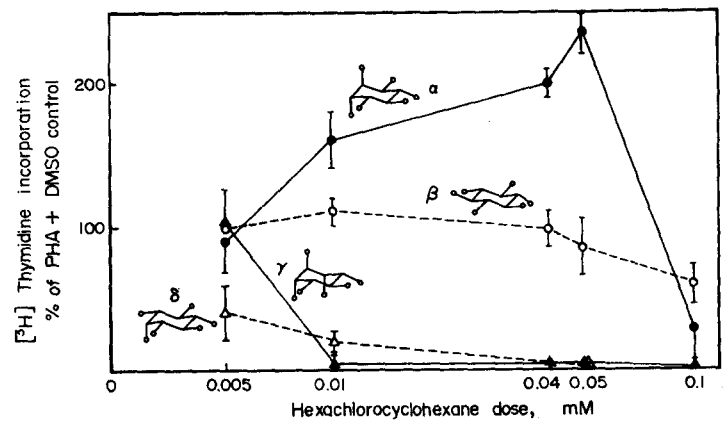

Fig. 5. Comparison of the effects of hexachlorocyclohexane isomers on PHA-mediated stimulation of [^{3}H]thymidine incorporation in lymphocytes. The indicated concentration of the α-, β-, γ- and δ-isomers of hexachlorocyclohexane was added to PHA-treated cultures of lymphocytes at time zero. The cultures were then pulsed with [^{3}H]thymidine from 48 to 50 hr. The data are expressed as per cent of the PHA + DMSO control. Key: (●——●) αHCH, (○---○) βHCH, (▲——▲) γHCH and (△---△) δHCH.

vate membrane metabolism. While the initial events do not depend on the synthesis of new RNA and protein species, they lead rapidly to an activation of these processes and the progressive expression of genes which carry the cell through G_1 into a replication response (i.e. S phase). Our working concept in studying this progression is that activation of specific regions of the cell membrane releases specific proteins for activation and transport to specific nuclear sites where they act as inducers. It is also our view that cells, according to their phenotypes, may require specific combinations of inducers in order to effect a replicative response and that the cell membranes may play a role in the summation and integration of mitogenic stimuli [10].

Our previous studies in human lymphocytes had revealed that inositol phosphatide turnover in lymphocyte membranes was strikingly stimulated in the first few minutes of PHA action [1] and that γHCH, an inhibitor of CDP-diglyceride-inositol transferase, blocked both the initial acceleration of inositol phosphatide metabolism and the subsequent events associated with the lymphocyte-transforming action of PHA [6]. In the present studies with bovine lymphocytes, γHCH was found again to inhibit the PHA mitogenic response; however, the delta-isomer was even more inhibitory. This finding is in agreement with the observations of Hokin and Brown [5] who showed that δHCH had a profound effect on phosphatide synthesis in general in guinea pig cerebral cortex slices whereas γHCH was specifically inhibitory to sites of inositol phosphatide synthesis which required activation by acetylcholine. Their studies point to the existence of restricted sites of phosphatidyl inositol metabolism and suggest that susceptibility of such sites to γHCH prior to activation of acetylcholine might be due to a deficiency of CDP-diglyceride, a protective substrate of the CDP-diglyceride-inositol transferase. The possibility that similar restricted sites may play an important role in the metabolic restraint of small lymphocytes is inferred from our present and past studies. In such a case, knowledge of the restricted sites in lymphocytes of different phenotypes may provide a basis for selective control of lymphocyte activity in leukemia, autoimmune, and certain immune deficiency states.

In these studies, the α-isomer (αHCH) proved to be unusual in that it was a co-mitogen in PHA-treated cells. This effect seems to override a limited inhibitory action which it has in common at higher doses with the γ- and δ-isomers. The observations that the co-mitogenic action could be blocked by SKF-525A, an inhibitor of mixed oxygenase function in the endoplasmic reticulum, suggests that αHCH may have to be metabolized to obtain the co-mitogenic action. The alternate possibility must be considered that αHCH induces or activates a SKF-525A-sensitive system which is necessary to the mitogenic response. However, with respect to metabolites, Grover and Sims [11] have proposed that the metabolism of hexachlorocyclohexanes involves dehydrochlorination and intermediary epoxide formation. Our observations that only the αHCH exhibits co-mitogenic activity suggests that spatial relationships of the chlorine atoms may play an important role in forming the hypothetical co-mitogenic derivative or in the subsequent action of this product. A search for highly active metabolites of αHCH is planned.

The present studies of HCH action in cultured lymphocytes provide an interesting corollary to their effects on hepatic tissue in living animals. Administration of αHCH to rats leads to enlargement of the liver and up to a 10-fold increase in the incorporation of labeled thymidine into hepatic DNA [12]. SKF-525A, as in the case of lymphocytes, prevents the αHCH stimulation of DNA synthesis in rat hepatocytes [13]. However, the situation may differ somewhat in rat liver since Schulte-Hermann *et al.* [14] have recently presented evidence that the SKF-525A inhibition *in vivo* of the hepatocyte response to αHCH is not due to an early blocking of the metabolic activation of αHCH, but rather reflects a general SKF-525A sensitivity that occurs 0–2 hr before the G_1/S transition. Such a late action for SKF-525A is

not apparent in bovine lymphocytes, since the delayed addition of SKF-525A has no effect on the PHA response alone or on the co-mitogenic action of αHCH.

In connection with the possible formation of a co-mitogenically active metabolite of αHCH by an SKF-525A-sensitive system, it should be recalled that αHCH is both an inducer of mixed-function oxygenase activity in liver [15–17] and a weak hepatic carcinogen in rats [18]. In this situation, the α-isomer is again unusual; Ito *et al.* [19] have recently reported the induction of hepatocellular carcinomas by high doses of αHCH, while the other isomers were oncogenically inactive. Nagasaki *et al.* [20] have also reported studies in which the α-, β-, γ- and δ-isomers of HCH were tested separately in mice. In this case, multiple liver tumors were found in all animals fed αHCH, but no lesions were produced with the other three isomers. Subsequently, β- and γHCH have been shown to have limited carcinogenic potential in mice [21]. From these studies, rats appear to be much more refractory to the carcinogenic action of the hexachlorocyclohexanes. It is also clear from structural considerations that the sterochemistry of a single chlorine group plays a profound role in determining the biological activity of αHCH. Whether the mitogenic (or co-mitogenic) activity of αHCH in the rodent liver is coupled to the chemistry of the carcinogenic action of αHCH remains to be determined.

An interesting aspect of the present experiments was the observation that αHCH prevents the mitogenic response (i.e. increased thymidine incorporation) associated with the mixed culture of genetically dissimilar lymphocytes. While this action may stem from the antimitogenic activity it shares at higher doseages with the γ- and δ-isomers, it appears that αHCH may be more selective than the other two isomers. Accordingly, αHCH promises to be a valuable tool in the elucidation of the molecular processes underlying mixed-lymphocyte responses and immunological rejection reactions in general. This aspect of αHCH action will be described in greater detail in a subsequent communication.

Finally, the requirement for concurrent PHA treatment and the necessity to have αHCH present during only the early hours of the PHA treatment suggests that transitory factors or lymphocyte states having their origin in the recent immunologic experience of the animal may be involved in the co-mitogenic action of αHCH. In this connection, we noted that lymphocytes from cows which have been exposed to severe weather (i.e. subzero temperatures) respond mitogenically to lower doses of PHA and were less responsive to αHCH. The possibility that previous adrenal stress or the altered levels of other hormones may be important in regulating the mitogenic responsiveness of lymphocytes appears likely. Accordingly, αHCH may play a further useful role in identifying factors *in vivo* which influence lymphocyte growth and differention.

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REFERENCES

1. D. B. Fisher and G. C. Mueller, *Biochim. biophys. Acta* **248**, 434 (1971).
2. G. C. Mueller and M. LeMahieu, *Biochim. biophys. Acta* **114**, 100 (1966).
3. D. C. Tormey and G. C. Mueller, *Blood* **26**, 569 (1965).
4. A. A. MacKinney, Jr., F. Stohlman, Jr. and G. Brecher, *Blood* **19**, 349 (1962).
5. M. R. Hokin and D. F. Brown, *J. Neurochem.* **16**, 475 (1969).
6. D. B. Fisher and G. C. Mueller, *Biochem. Pharmac.* **20**, 2515 (1971).
7. G. C. Mueller, K. Kajiwara, E. Stubblefield and R. R. Rueckert, *Cancer Res.* **22**, 1084 (1962).
8. A. M. Mastro and G. C. Mueller, *Expl Cell Res.* **88**, 40 (1974).
9. L. R. Thompson and G. C. Mueller, *Biochim. biophys. Acta* **378**, 344 (1975).
10. G. C. Mueller, *The Cell Cycle and Cancer*, pp. 269–307. Marcel Dekker, New York (1971).
11. P. L. Grover and P. Sims, *Biochem. J.* **96**, 521 (1965).
12. I. Schlicht, W. Koransky, S. Magour and R. Schulte-Hermann, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **261**, 26 (1968).
13. R. Schulte-Hermann, I. Schlicht, W. Koransky, C. Leberl, C. Eulenstedt and M. Zimek, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **223**, 109 (1972).
14. R. Schulte-Hermann, C. Leberl and I. Ruberg, *Biochim. biophys. Acta* **447**, 413 (1976).
15. W. Koransky, S. Magour, H. J. Merker, I. Schlicht and R. Schulte-Hermann, *Proc. Third Int. Pharmac. Meeting (Sao Paulo)* **4**, 55 (1966).
16. W. Koransky, S. Magour, G. Noack and R. Schulte-Hermann, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **263**, 281 (1969).
17. J. Seifert and J. Vacha, *Chem. Biol. Interact.* **14**, 359 (1976).
18. *IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Man*, Vol. 5, pp. 47–94. Lyon, France (1974).
19. N. Ito, H. Nagasaki, H. Aoe, S. Sugihara, Y. Miyata, M. Arai and T. Shirai, *J. Natn. Cancer Inst.* **54**, 801 (1975).
20. H. Nagasaki, S. Tomii, T. Mega, M. Marugami and N. Ito, *Gann* **63**, 393 (1972).
21. E. Thorpe and A. I. T. Walker, *Fd Cosmet. Toxic.* **11**, 433 (1973).