Human Peripheral Blood in Tissue Culture and the Action of Phytohemagglutinin

In the past few years there has been increased interest in the study of human peripheral blood leucocytes in tissue culture. During these studies the hemagglutinating substance[1], phytohemagglutinin (PHA), was found to induce mitosis[2] in these cultures. This substance, extracted from the red kidney bean[1], is now widely used in the culture of leucocytes for chromosome analysis.

Much attention is now being focused on the action of PHA. There is evidence that the human, small lymphocyte[3–10] is the cell transformed *in vitro* in the presence of this substance into a blastoid cell capable of division. This paper will draw attention to the correlation between PHA and 'blastoid' changes of mononuclear cells leading to cell division in short term cultures of normal and leukemic, peripheral blood leucocytes. Experiments will also be presented which were designed to provide information concerning the mechanism by which PHA induces this blastoid transformation.

Materials and Methods

Method of culturing peripheral blood. The culture method is a modification of that used by several investigators[2,8]. Peripheral blood was collected in an evacuated glass tube (the Becton-Dickinson Vacutainer) containing either powdered heparin or a heparin solution (200 U per 10 ml of blood) which contained 0.9% benzyl alcohol as a preservative. The blood was kept at 4°C for 1 h during which time some sedimentation of erythrocytes occurred. (To obtain adequate sedimentation of erythrocytes in cord blood, the cord blood was diluted with autologous cord plasma prior to this hour in the cold.) Thereafter, to obtain more complete separation of the erythrocytes from the leukocyte-rich plasma, the blood was centrifuged at 300 to 500 rpm for 5–12 min. The leucocyte-rich, erythrocyte-poor, supernatant plasma was then collected. If this supernatant contained excessive numbers of leucocytes, it was diluted with autologous, cell-free plasma, so that the final culture fluid contained 0.8–2.2 million leucocytes per ml. The final culture fluid was made by adding one part of this leucocyte-rich plasma to four parts of tissue culture Medium 199 (Baltimore Biological Laboratories) containing sodium bicarbonate. Antibiotics had been added to the Medium 199 so that their concentrations in each ml of the final culture fluid were 70 μg of streptomycin, 70 U of penicillin, and 35 U of mycostatin. The desired amounts of PHA were added either to the Medium 199 or to the final culture fluid.

The PHA used, Bacto-Phytohemagglutinin M (PHA-M), is prepared by Difco Laboratories, Detroit (Michigan), according to the procedure of RIGAS and OSGOOD[1]. Each vial of desiccated PHA-M was rehydrated prior to use with 5 ml of water or saline, stored at 4°C, and discarded if not used within one week.

One ml aliquots of the final culture fluid were placed in screw-capped tubes, which were then maintained in a stationary, vertical position at 35.0°–36.0°C. In some instances cultures were grown directly on cover-slips contained in Leighton culture tubes. After the desired period of incubation, the cells were obtained for examination from the screw-capped tubes after gentle centrifugation. The centrifuged cells were redispersed in a small volume of supernatant culture fluid by repeated aspirations in a pipette, smeared on coverslips, air dried, and stained with Wright's Stain.

Sources of leucocytes. (a) Normal leucocytes: Leucocytes were obtained from 77 samples of peripheral blood from 30 adults with no known hematological disease.

(b) Leukemic leucocytes: Cultures were made from the peripheral blood of three patients with acute leukemia. One adult had the clinical diagnosis of acute myelogenous leukemia. Another adult had an acute blastic leukemia, the exact type of which could not be determined. The third patient, an eight-year-old boy, had acute lymphoblastic leukemia. Cultures were made also of the peripheral blood from three patients diagnosed clinically as having chronic lymphocytic leukemia (CLL). Their white blood-cell counts were above 90 000 cells per mm^3 of peripheral blood, and in each case more than 93% of these cells had the morphology of lymphocytes. None of the six patients with leukemia had been treated with anti-leukemic or steroid drugs.

(c) Leucocytes from individuals with impaired immune reactions: Two specimens of cord blood were cultured. These specimens were obtained by puncture of the umbilical cord vessels of placentas within 8 min after their delivery. Leucocytes were cultured from the blood of two brothers who had a form of congenital, idiopathic hypo-γ-globulinemia. Histories, clinical and laboratory findings of these two patients have been reported by HENLEY[11].

Method for removal of the erythrocyte-agglutinating activity of PHA-M. Two ml of saline-reconstituted PHA-M were mixed with 2 ml of saline-washed, packed red cells obtained from the person whose leucocytes were to be cultured. After 25 min at 4°C the mixture was centrifuged and the supernatant removed and mixed with a second aliquot (0.5 ml) of the packed cells. After 10 min at 4°C this mixture was centrifuged, and the resulting supernatant passed through a 0.45 μ pore-size, millipore filter to remove any remaining blood cells. The resulting, cell-free filtrate is referred to as 'erythrocyte-treated PHA-M'. Titration of erythrocyte-agglutinating activity was performed essentially as described by SALK[12].

Results

The production of blastoid cells in cultures of normal leucocytes by PHA-M. The general effects of PHA on normal peripheral blood cells in tissue culture has been described by several investigators[2,5,6,8,13,14] and will not be described in detail herein except as they apply to the formation of blastoid cells and are necessary as a frame of reference for comparison with the results reported below. The larger blastoid cells formed possess a round or

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oval nucleus with one or more prominent nucleoli (Figures 1a and 1c). The basophilic, nongranular cytoplasm often contains clear vacuoles (Figure 1c). These large, blastoid cells can be observed in mitosis after the 48th h of culture. Smaller blastoid cells are also seen. These cells appear in the PHA-M-treated cultures prior to the appearance of the larger cells and possibly represent transitional stages in the transformation of the small lymphocyte to the larger blastoid cells. Many of the large and small blastoid cells are found in clusters (Figures 1a and 2c).

On the 3rd day of culture of normal peripheral blood not exposed to PHA-M, less than 0.5% of the mononuclear cells present are blastoid. The small, unchanged lymphocytes are readily apparent (Figures 1b and 2a).

Effects of varying the concentration of PHA-M are striking. For example, with a concentration of 0.01 ml per 5 ml of culture fluid 1% of the mononuclear cells were blastoid after 69 h in culture. With increasing concentrations of PHA-M the percentage of blastoid cells increased, so that with a concentration of 0.20 ml per 5 ml of culture fluid, more than 70% of the mononuclear cells present at 69 h were blastoid (Figure 2). This transformation occurred with a corresponding decrease in the percentage of small lymphocytes present.

PHA-M and leukemic blast cells. In cultures from two of the three patients with acute leukemia mitotic figures were seen in the first 48 h of culture in the absence of PHA-M. During this period the number of cells with mitotic figures was not noticeably increased by PHA-M in a concentration of 0.05 ml per 5 ml of culture fluid. In the cultures from the other patient no mitotic figures were seen during the first 48 h of culture even in the presence of PHA-M.

PHA-M and chronic lymphocytic leukemia cells. All the short term cultures of chronic lymphocytic leukemia (CLL) leucocytes showed a markedly reduced response to PHA-M. For example, cultures made from two of these patients were tested with several concentrations of PHA-M. Even at the highest concentrations employed (0.25 and 0.50 ml per 5 ml of culture fluid), less than 3% of the cells had become blastoid during the first $3^1/_2$ days in culture. It should be noted, however, that the large blastoid cells formed in cultures of CLL blood in the presence of PHA-M (Figure 3c) appeared morphologically similar to the blastoid cells formed in cultures of normal blood.

In order to determine if PHA-M-containing, CLL culture fluid could transform normal lymphocytes into the characteristic blastoid cells, cell-free millipore filtrates of these fluids were obtained. These filtrates were added to normal lymphocytes from PHA-M-free cultures and, in 3 days, had induced a characteristic blastoid transformation so that more than 70% of the mononuclear cells present were blastoid. No transformation occurred if the filtrates were obtained from PHA-M-free, CLL cultures.

Cell-free, millipore filtrates from PHA-M-containing cultures of normal blood, in which blastoid transformation had occurred, were added to CLL leucocytes which had been cultured without PHA-M. On the 3rd day in the presence of these filtrates more than 97% of the CLL lymphocytes were small and had not become blastoid.

PHA-M and leucocytes from individuals with impaired immune reactions. The leucocyte cultures from cord blood and from blood of the patients with hypo-γ-globulinemia responded normally to PHA-M as judged by the morphology of the blastoid cells formed, the concentrations of PHA-M required to transform more than 70% of the lymphocytes into blastoid cells, and the time required for these responses.

Erythrocyte-treated PHA-M and normal leucocytes. Prior to its exposure to the packed cells, the PHA-M used in this study produced agglutination of erythrocytes in a dilution of 1:320. Erythrocyte-treated PHA-M did not agglutinate (even at a 1:2 dilution) the erythrocytes of the person whose leucocytes were cultured. However, the blastogenic and mitogenic activity of erythrocyte-treated PHA-M remained essentially unchanged. Over 60% of the mononuclear cells became blastoid in a 69 h culture exposed to 0.20 ml of the erythrocyte-treated PHA-M per 5 ml of culture fluid (Figure 4). Erythrocyte agglutination

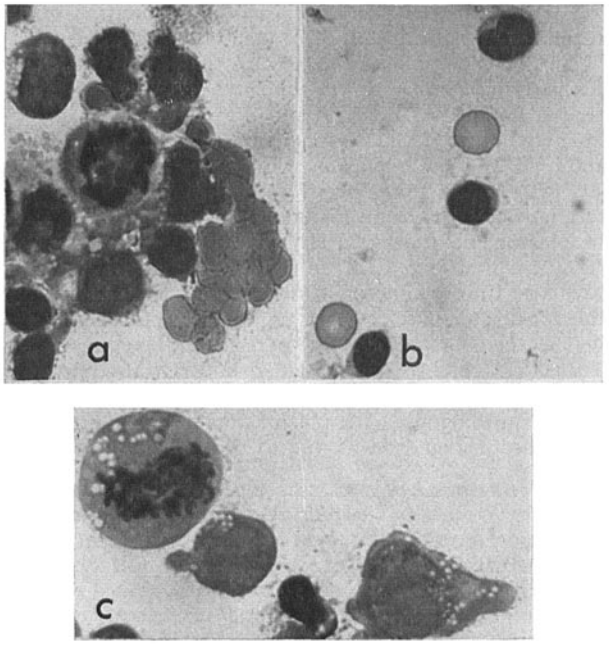

Fig. 1. Normal leucocytes in 69 h cultures. a and c, with 0.20 ml of PHA-M per 5 ml of culture fluid; b, without PHA-M. In 'a' note the clustering of the blastoid cells around the cell in mitosis. Note also the large clump of agglutinated erythrocytes in the lower right of the field in 'a' (× 810).

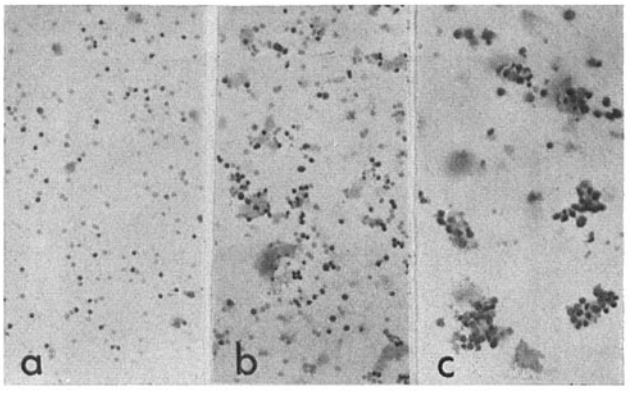

Fig. 2. Effect of different concentrations of PHA-M in 69 h cultures. a, without PHA-M; b, with 0.05 ml of PHA-M per 5 ml of culture fluid; and c, with 0.20 ml of PHA-M per 5 ml of culture fluid. The small, unchanged lymphocytes in 'a' appear as tiny, solid, black dots amidst the lighter staining erythrocytes. The magnification, × 90, is the same throughout.

was absent in these cultures (Figure 4b) in contrast to the clump of agglutinated erythrocytes which can be seen in Figure 1a. It is of interest, however, that in the cultures with erythrocyte-treated PHA-M the blastoid cells still formed clusters.

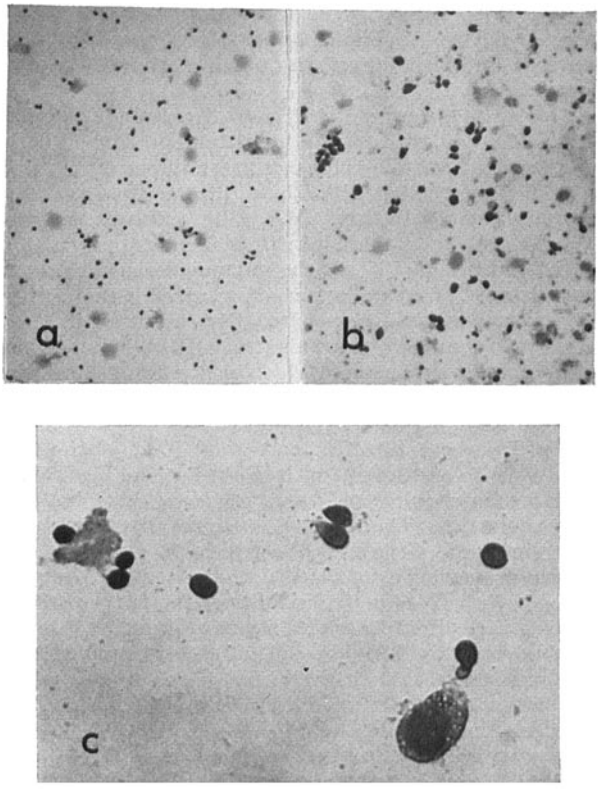

Fig. 3. a and b, low magnification views (× 90) of 82 h cultures with 0.05 ml of PHA-M per 5 ml of culture fluid: a, chronic lymphocytic leukemia (CLL) leucocytes; b, normal leucocytes. Note the small, unchanged lymphocytes in 'a'. The culture of normal leucocytes 'b' has many blastoid cells and was made at the same time as the culture of CLL leucocytes, under the same conditions, and with the same batch of PHA-M. c, high magnification view (× 600) of another CLL patient's leucocytes in a 73 h culture with 0.25 ml of PHA-M per 5 ml of culture fluid. Note the single blastoid cell in the presence of several small lymphocytes.

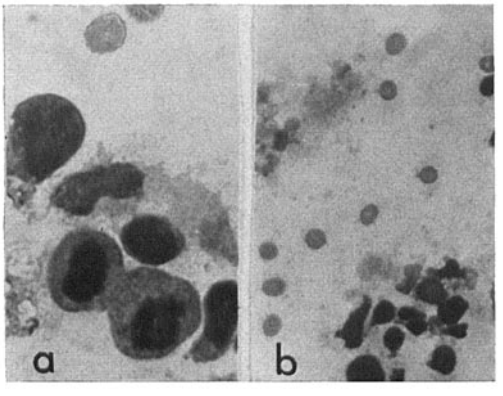

Fig. 4. 69 h culture of normal leucocytes with 0.20 ml of erythrocyte-treated PHA-M. a, × 810. Note that one of the blastoid cells is dividing. b, different field, × 360. Note the unagglutinated erythrocytes above the group of blastoid cells.

Discussion

Several previous studies[3–10] have indicated that the small lymphocyte of human, peripheral blood is transformed into a blastoid cell in the presence of PHA *in vitro*. The results of the present study are consistent with this view, for, during the first 48 h of culture, blastoid cells appeared in increasing numbers while the number of small lymphocytes decreased. This change occurred without evidence of cell division prior to the 48th hour or of destruction of the latter cell. It appears, therefore, that more than 70% of the normal, small lymphocytes from human, peripheral blood are transformed into blastoid cells *in vitro* in the presence of adequate concentrations of PHA-M. It is not known, however, whether the monocyte and the large and medium lymphocytes are transformed into blastoid cells or made to divide by PHA-M.

Investigators have evaluated the effect of PHA on mononuclear leucocytes by determining the frequency of cell division (i.e. the mitotic index)[2,6,7] or the rate and extent of incorporation of radioactive precursors into nucleic acids[3,6,14–16]. In the experiments with PHA-M described herein, the 'blastogenic' activity of PHA-M has been stressed for the following reasons: (1) the development of blastoid cells (blastogenesis) precedes and is a prerequisite for the appearance of mitotic figures; (2) the degree of blastogenesis is easily evaluated (see Figure 2); and (3) by focusing on the first, observable effects of PHA-M, it is hoped that attention will be directed toward the basic mechanisms initiating blastogenesis and cell division.

Several investigators have reported that no blastoid cells or cells in division developed in PHA-treated cultures of peripheral blood from some patients with CLL[17–19]. However, ELVES and WILKINSON[7] reported that the number of blastoid and dividing cells in PHA-treated cultures of CLL blood did not vary significantly from that of PHA-treated cultures of normal blood. Other investigators[20], who have studied the chromosomes in PHA-containing CLL cultures, made no mention of the frequency of cell division or of blastoid cells formed.

The present studies demonstrate that more than 97% of the lymphocytes in the cultures of the CLL blood studied herein did not respond normally to the blastogenic effect of PHA-M. It seems unlikely that there was a factor in the CLL plasma which prevented the CLL patients' lymphocytes from reacting, for lymphocytes from normal individuals responded in a normal manner to the blastogenic effect of PHA-M when transferred to the PHA-M-containing, CLL culture fluid. This result indicates also that the factor in the PHA-M which is responsible for blastogenesis was not significantly inactivated by the CLL cells or plasma. The persisting unresponsiveness of the CLL cells when suspended in PHA-M-containing, normal, culture fluid (20% of which was normal plasma instead of autologous, CLL plasma) makes it unlikely that the unresponsiveness was due to the lack of a necessary

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factor in the CLL plasma, for such a factor would probably have been present in the transferred fluid.

It should be noted that in at least one respect the conditions of the culture of CLL cells are different from those of the culture of normal cells. Because more than 93% of the leucocytes in the CLL blood were lymphocytes, cultures therefrom have relatively few polymorphonuclear neutrophils and monocytes. However, in the experiments of CARSTAIRS[5] and of others[10,18,21] most of the neutrophils and monocytes were removed prior to the culturing of normal cells without any noticeable effect upon the blastogenic and mitogenic effects of PHA.

The above data suggest that more than 97% of the lymphocytes in the CLL blood cultures studied herein were *inherently* unable to respond within $3^1/_2$ days in a normal manner to the blastogenic effect of PHA-M. The few large blastoid cells formed in the presence of PHA-M in these cultures may have been derived, for example, from either the monocytes or a small population of normal lymphocytes, or both. It is possible, therefore, that there were two types of lymphocytes in the CLL blood: the leukemic lymphocytes, comprising more than 97% of the total lymphocytes, which did not respond within $3^1/_2$ days to PHA-M, and the normal lymphocytes, comprising less than 3% of the total lymphocytes, which did react to PHA-M. If this hypothesis is true, it must be concluded that the chromosomes of the abnormal cells of these CLL patients would never have been seen in these short-term peripheral blood cultures. The chromosomes seen would have been those of normal cells.

The blasts in the peripheral blood in two of the three cases of acute leukemia studied herein divided in tissue culture even in the absence of PHA-M. Similar findings have been reported by previous investigators[7,22,23]. There is no evidence that PHA-M increases the number of mitoses in these leukemic blast cells. However, it is reasonable to assume that any normal lymphocytes which might be present in cultures of peripheral blood leucocytes from patients with leukemia would eventually divide in the presence of PHA-M. These normal lymphocytes would contain normal karyotypes and, as already noted[24–26], might interfere with the determination of the frequency of a chromosomal abnormality present only in the leukemic cells. These considerations may account for some of the variation in the frequency with which the Ph[1] chromosome has been found in PHA-containing cultures of peripheral blood from patients with chronic myelogenous leukemia[24–26].

In studies which are designed to examine the chromosomes of the leukemic cell, it would be advisable, therefore, to avoid the mitogenic effect of the PHA on normal lymphocytes. This may be done by omitting PHA completely. The cells found in division in the absence of PHA would, then, probably be only the leukemic cells. Since, however, in certain leukemias, for example, 'familial', chronic lymphocytic leukemia[20] and the leukemias associated with mongolism[25], all cells possess a chromosomal abnormality, the mitogenic effect of PHA is advantageous, for it causes lymphocytes to divide and thereby provides cells in mitosis which will reveal the chromosomal abnormality. But it must be noted that even in the latter cases the leukemic cell may have a karyotype which differs from that of the non-leukemic cells[27,28]. Thus, peripheral blood cultures with and without PHA should be used routinely.

Mechanism of the induction of blastogenesis by PHA-M. It has been suggested that the mitogenic activity of PHA might be related to an immunologic mechanism[7,14,17,29,30]. This suggestion is supported by the findings that blastoid cells are formed from a sensitized person's peripheral, blood leucocytes when they are exposed to the specific, sensitizing antigen *in vitro*[29–31]. In order to test this suggestion, the blastogenic effect of PHA-M on cultures of cord blood and blood from patients with hypo-γ-globulinemia was studied. Since the two hypo-γ-globulinemic patients had impaired production of certain antibodies[11], it was hypothesized that their blood cells might fail to react normally to PHA-M. As reported above, no impaired reaction was found. These studies should be repeated in other patients who have more severe degrees, and different types, of hypo-γ-globulinemia.

More than 70% of the mononuclear cells in the cord blood cultures were blastoid by the 72nd hour of culture in the presence of adequate concentrations of PHA-M. This response is of great interest since certain immunological mechanisms[32–34], but possibly not all[34], seem to be markedly impaired in the newborn. However, it has recently been demonstrated that Atrabine-labeled maternal lymphocytes pass from the maternal blood into cord blood[35]. It must be noted, therefore, that some of the cells in cord blood which reacted to the blastogenic factor of PHA-M might have been cells of maternal origin. But it seems likely that some, if not the majority, of the blastoid cells formed were of fetal origin. The maternal cells, in contact with cells of fetal origin, may have transferred some form of immunological responsiveness to the latter cells, perhaps in a manner analogous to the transfer of delayed hypersensitivity in humans[36]. It should be noted that the fetal or maternal origin of the cells in cord blood which divide *in vitro* in the presence of PHA-M might be determined by culturing the cord blood from a male child and by determining the sex chromosome complement (XX or XY) present in the dividing cells.

It should be noted also that several investigators have presented evidence indicating a possible relationship between the mitogenic effect of PHA and delayed-type

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hypersensitivity reactions[4,17,29]. The existence of such a relationship might be tested by determining if the response to PHA-M is impaired in cultures of leucocytes from patients with active sarcoidosis, a disease in which there is often an impairment of delayed skin reactions to a variety of antigens[37].

The relationship between the mitogenic and hemagglutinating effects of PHA is of considerable interest. There is evidence that the hemagglutinating activity can be present in the absence of mitogenic activity[38]. The possibility that the mitogenic activity could exist in the absence of hemagglutinating activity was supported by the work of GENEST[39]. He used a semi-purified PHA preparation and stated that '...filtration through a Seitz filter (disc S-1) inhibits the agglutinating capacity but not the mitogenic activity of our vacuum desiccated extracts. This inhibition does not appear following filtration of lyophilized material'. No further details of this phenomenon were presented.

In the studies performed herein, the erythrocyte agglutinating activity of PHA-M has been removed, but the blastogenic and mitogenic activity has been retained. Presumably, the hemagglutinating factor has been adsorbed to or otherwise inactivated by the erythrocytes during their agglutination. The method used here was suggested by the observation of RIGAS and OSGOOD[1] that the agglutinating activity of the supernatant of their PHA decreased during the process of hemagglutination. While this paper was in preparation, it was reported that substances with 'leucocyte growth factor activity'[40] and mitogenic activity[31] had been obtained free of erythrocyte agglutinating activity.

The data presented above strongly suggests that the erythrocyte-agglutinating and blastogenic activities of PHA-M reside in separate molecules. Because of the method of preparation of PHA-M[1], it is quite possible that these molecules are proteins. In this regard it should be noted that PHA-M has been shown to contain many proteins[41]. Further characterization and purification of the active factors of PHA-M by chemical, electrophoretic and immunologic means, would be of great interest[42].

Résumé. Phytohémagglutinin a transformé en cultures brèves plus de 70% des petits lymphocytes de personnes normales, de nouveau-nés et d'hypo-γ-globulinémiques en cellules blastoïdes, mais moins de 3% des lymphocytes de malades avec leucémie lymphatique chronique. L'action blastogénique existe dans l'absence erythrocyte-agglutinante. On discute la signification de ces découvertes.

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[42] *Acknowledgments.* I am greatly indebted to Mrs. B. SHORE and to Professor A. WACHTEL for their helpful advice and encouragement throughout.
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CONGRESSUS

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Persons interested in participating are kindly requested to apply not later than June 30, 1963, to the following address: The Organizing Committee, 3rd European Regional Conference on Electron Microscopy, Prague 1964, Albertov 4, Prague 2 (Czechoslovakia).

Niederlande

Holländisch-Deutsche Analytikertagung im Frühjahr 1964

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