Preparation and Purification of Tritiated Phytohemagglutinin and Studies of Cellular Localization in Human Leukocyte Cultures

By Robert A. Conard and Charles F. Demoise

The discovery of the mitogenic action of the bean (Phaseolus vulgaris) extract, phytohemagglutinin (PHA), in lymphocyte cultures by Nowell has opened up new avenues of research. Many studies have been reported concerning the morphological and biochemical changes associated with the blast-like changes of the lymphocytes leading to replication, for the exact mechanism of action of PHA has remained obscure. It has been suggested that PHA acts by precipitation of a mitogenic factor in the serum, attachment of the antigen to the leukocyte, action on the cell membranes to permit entrance of material necessary for cell replication, and agglutination of leukocytes.

It is generally believed that PHA evokes a nonspecific blastogenic response in lymphocytes, in view of the fact that a much larger number of lymphocytes of the peripheral blood are stimulated than is noted in response to specific antigens (such as tuberculin, tetanus, typhoid, and pertussis). The transformation also occurs earlier in PHA-stimulated cultures as opposed to those using specific antigens. It is controversial whether or not PHA induces antibody production. The endoplasmic reticulum is much more poorly organized in the lymphocytes transformed by PHA than is seen in antibody-producing plasma cells. However, some investigators report antibody production in PHA-stimulated lymphocytes and immunofluorescent studies have revealed intracellular localization of the tag.

In a previous report, Conard reported intracellular localization in cultured human leukocytes of unpurified radioactive PHA obtained from red kidney beans grown in tritiated nutrient media. Autoradiograms showed that the labeled material was present in both nuclei and cytoplasm. The present paper reports the isolation and purification of tritiated extracts with greater specific radioactivity and more precise intracellular localization of the labeled material.

Materials and Methods

Growth of Radioactive Beans

A preliminary attempt to make radioactive extracts of beans (Phaseolus vulgaris) by exposure to tritium gas under vacuum with and without catalysts had failed. Mitogenic activity was invariably lost in the processing. Consequently, growth of the beans in...
tritiated water added to nutrient media (hydroponic growth) was carried out in a greenhouse within a closed plexiglass box with a special closed system of ventilation to avoid radioactive contamination (Fig. 1). Varying amounts of tritiated water were used to give final concentrations from 0.5 to 11 Ci/L, with different lots of beans. Damage was produced in plants exposed at too young an age or after too long an exposure to higher doses. By trial and error it was found that highest specific-activity extracts were obtained from exposure of plants with mature beans for one to two weeks to higher concentrations of $^3$H$_2$O.

**Extraction and Purification of Radioactive Phytohemagglutinin ($^3$HPHA)**

A modification of the method of Takahashi et al.\textsuperscript{10} was used to extract and purify a labeled mitogenic substance from the beans. The procedure was carried out at 4°C. A crude water extract was acidified (pH 4.6) and centrifuged in order to obtain a clear supernatant. Ammonium sulfate was then added to the supernatant until the saturation point was reached and the resulting precipitate dialyzed against distilled water for 24 hr. and lyophilized. The lyophilized powder was dissolved in 5 mM phosphate buffer (pH 6.8) and applied to a DEAE column (3 cm. $\times$ 36 cm.). This was washed free of carbohydrate with 600 ml. of the same buffer. Gradient elution was initiated by passing 0.2 M phosphate buffer (pH 6.8) into a closed mixing chamber containing 800 ml. of the starting buffer. 1-ml. fractions were collected and monitored at 280 nm. The first major absorbing fraction was lyophilized and dissolved in 5 mM citrate buffer (pH 3.8), and further purified by passage through a CM-52 column (1 cm. $\times$ 30 cm.). The column was washed with 200 ml. of buffer at which point gradient elution was begun by passing 0.1 M phosphate buffer (pH 6.8) into a closed mixing vessel containing 500 ml. of the starting buffer. The first major fraction absorbing at 280 nm. was then lyophilized, dissolved in 5 mM phosphate buffer (pH 6.8), and filtered through a Sephadex G-100 column (1 cm. $\times$ 30 cm.). The first fraction obtained at void volume was used for testing purposes. The lyophilized powders from each stage of purification were assayed for radioactivity by liquid scintillation counting in a xylene-alcohol-water system.

Microelectrophoresis was performed on each column fraction using strips of cellulose acetate (Phoroslide) and a Millipore cell (Millipore Corp., New Bedford, Mass.). Barbitol buffer (pH 8.6, ionic strength 0.075) was used with a run separation of 17 min. at 100 v. Ponceau-S dye was used to stain the protein bands. Autoradiograms were also made of the strips.

Mitogenicity of the column fractions was tested in leukocyte cultures, as described below, using concentrations of 2–60 μg./ml. of culture.

**Localization of Radioactive Label.** Human leukocyte cultures were prepared by separating leukocytes from the blood by sedimentation and centrifugation, and grown in plastic
vessels at a concentration of $10^6$ cells/ml. in Eagles' Minimum Essential media supplemented with 1 per cent glutamine, 15 per cent fetal calf serum, and penicillin (100 U./ml.) and streptomycin (0.1 mg./ml.) at 37° C. $^3$HPHA (either CM-52 or Sephadex extract) usually at 4 μg./ml. was then added. The cells were harvested at various times, treated with proteolytic enzyme (pronase) and a cytoplasmic stripping agent (cetrimid) as described by Stewart and Ingram. The nuclei were counted and sized with a Coulter electronic counter (Model A). Previous experiments had shown that nuclei larger than 47 cu. μ were largely blast cells and mitogenic activity was expressed as per cent transformation. At 6, 24, 36, 48, 60, 72, and 96 hr. after addition of $^3$HPHA, cultures were counted, centrifuged, and smears and chromosome preparations made, using a modification of the technique of Hungerford et al. Mitosis was arrested at metaphase with vinblastine sulfate (0.0005 μg./ml. of culture). Autoradiograms of the slides were prepared by dipping them in Kodak nuclear emulsion, type NTB, developed at varying times (40–80 days), and stained with Giemsa.

Subcellular fractionation (nuclei, mitochondria, and microsome fractions) was obtained by differential centrifugation after the method of Fishman and Silverman. These fractions were monitored for radioactivity by gas-phase proportional counting. Selective removal of ribonucleic acid from fixed smears with 10 per cent perchloric acid was carried out according to the method of Baserga and Kisielski. The same procedure was carried out on cultures flash labeled with tritiated cytidine in order to verify RNA removal. The treated slides, along with control slides, were then dipped for autoradiography and examined after suitable exposure.

Ribonucleic acid was also extracted from cell pellets of cultures according to a modification of the phenol procedure described by Barlow and Mathias. In the same manner, deoxyribonucleic acid was removed by increasing the molarity of the Tris-HCl buffer to 1 M and omitting the sodium dodecyl sulfate solution. The resulting RNA and DNA extracts were then assayed for radioactivity by the gas-phase proportional technique.

**RESULTS**

**Purification of the Bean Extract.** The major absorbing fraction obtained after column chromatography of extracts from five separate lots of beans grown in tritiated media were quite similar to one another. Fractionation with DEAE, CM-52, and Sephadex G-100 columns showed increasing purification of the protein moiety, as shown in Fig. 2.

Specific activity of tritium label in different lots of beans is shown in Table

![Fig. 2.—Column chromatography purification. Second peak in Sephadex separation not mitogenic.](image-url)
Table 1.—Radioactivity of Column Fractions

<table>
<thead>
<tr>
<th>Lot</th>
<th>Tritiated $^3$H$_2$O of Growth Media (Ci/L.)</th>
<th>Ammonium Sulfate ($\mu$Ci/mg.)</th>
<th>Column 1 DEAE ((\mu)Ci/mg.)</th>
<th>Column 2 CM-52 ((\mu)Ci/mg.)</th>
<th>Sephadex G-100 ((\mu)Ci/mg.)</th>
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<tr>
<td>1</td>
<td>0.75</td>
<td>0.20</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.75</td>
<td>0.16</td>
<td>0.10</td>
<td>0.10</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>0.75–1.75</td>
<td>0.20</td>
<td>0.17</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>0.75–8.00</td>
<td>0.40</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>5</td>
<td>11.00</td>
<td>1.00</td>
<td>0.50</td>
<td>0.50</td>
<td>0.45</td>
</tr>
</tbody>
</table>

1. In all cases some loss of specific activity occurred in the column 1 (DEAE) step of purification. This was probably due to loss of the labeled carbohydrate fraction which may have had a higher specific activity, and also from loss of freely exchangeable tritium label.

When aliquots from each stage of purification were subjected to electropho-

Fig. 3.—Autoradiography of electrophoretic bands of column fractions.
Table 2.—Mitogenicity of Column Fractions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2 μg./ml.</th>
<th>Per cent Transformation 4 μg./ml.</th>
<th>20 μg./ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium Sulfate</td>
<td>26</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>DEAE Column</td>
<td>31</td>
<td>31</td>
<td>37</td>
</tr>
<tr>
<td>CM-52 Column</td>
<td>51</td>
<td>73</td>
<td>65</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>45</td>
<td>64</td>
<td>60</td>
</tr>
</tbody>
</table>

resis, and the resulting phoroslides autoradiographed, bands of radioactivity coincided with the protein bands showing maintenance of the label during the purification process. This is shown in Fig. 3.

Mitogenicity of each fraction was tested by measuring the per cent transformation. The mitogenicity of the several column fractions (Table 2) is indicated by the average per cent transformation of three cultures using three dilutions of extracts from several lots of beans.

There was a three-fold increase in mitogenicity between the ammonium sulfate fraction and the CM-52 at the optimum concentration (4 μg./ml. of culture) of the purified extract. Compared with commercial PHA-M (Difco, the purified extract showed about an 80-fold increase in activity on a weight

Fig. 4.—Cytoplasmic labeling of lymphocytes in 24-hr. culture treated with labeled PHA. Lymphocytes surround labeled cellular material near center of group (× 2200).

Fig. 5.—Labeled macrophages with two adjacent labeled lymphocytes. Label of red cells was not seen (× 2200).
basis. Exceeding this concentration resulted in decreased mitogenicity and increased toxicity as evidenced by cellular disruption. The reason for the slight loss in mitogenicity following Sephadex treatment is not known but may be due to some denaturation of the protein during this step. Secondary peaks from the various columns were radioactive but not mitogenic, and did not produce significant label of cells in culture.

**Autoradiographic Studies.** By examining grain distribution in autoradiograms at various times after the beginning of culture, the following chronological sequence of events was noted. Within a few hours labeling was seen in almost all polymorphonuclear leukocytes, many larger mononuclear cells, and some lymphocytes. This label appeared to be largely cytoplasmic. The red blood cells did not appear to be labeled at this time or later, and no label was ever noted which appeared to be on the cell membrane of the leukocytes. Marked agglutination and disintegration of many labeled cells were noted during the first 24 hr. Most recognizable polymorphonuclear cells had disappeared after the first day. Heavily labeled masses of disintegrated cells which took a basophilic stain were noted by 24 hr (Fig. 8). These masses were frequently surrounded by lymphocytes, with labeled material often

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**Fig. 6.—Group of blast cells in three day culture (× 1500).**

**Fig. 7.—Blast cell undergoing mitosis. Label largely cytoplasmic (× 2200).**
concentrated in a cytoplasmic elongation oriented toward the central mass (possibly the "Uropods" of McFarland et al.) (Fig. 4). Macrophages with phagocytized labeled material were frequently seen (Fig. 5). After 24 hr. enlarged lymphocytes with cytoplasmic label were numerous and were later followed by the appearance of labeled blast cells (Fig. 6–9). These cells had lightly stained, loosely knit nuclear material with prominent nucleoli and abundant basophilic cytoplasm. They were usually more heavily labeled than the untransformed lymphocytes.

Comparing density of grains over the nucleus with cytoplasm showed that the majority of the grains were over the cytoplasmic region even though the nuclei of these cells in most cases covered over half the cell area (Figs. 4–9). Nuclei stripped of cytoplasm by cetrimide treatment showed little label, though occasional cytoplasmic shreds clinging to the nucleus showed definite label. It is noteworthy that no consistent labeling of the nucleoli was seen. In chromosome preparations no label over the chromosomes was observed. Figure 8 shows lack of chromosomal labeling in a blast cell undergoing mitosis. Subcellular fractionation studies discussed below also indicated largely a cytoplasmic localization.

Fig. 8.—Labeled blast cell near heavily labeled amorphous mass of cellular material (X 2200).

Fig. 9.—Blast cell with cytoplasmic label showing footlike projection (uropod) (X 2200).
Table 3.—Radioactivity Analysis of Subcellular Fractions

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>32*(7695)†</td>
<td>37(37,000)</td>
<td>27(1590)</td>
<td>21(2790)</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>52 (12,748)</td>
<td>57(58,000)</td>
<td>67(3900)</td>
<td>64(8430)</td>
</tr>
<tr>
<td>Microsome</td>
<td>16 (3949)</td>
<td>6(6000)</td>
<td>6(330)</td>
<td>15(1920)</td>
</tr>
</tbody>
</table>

* Per cent of combined fractions.
† Gas-phase proportional counts (DPM).

Nucleic Acid Extraction. Cellular grain counts and their distribution were essentially the same in control and perchloric acid extracted smears. This could be interpreted to mean that the labeled material was not bound to the RNA since it was not removed by this procedure. (Cells labeled with tritiated cytidine when treated with perchloric acid lost most of their label.) Extracts of DNA and RNA from cell pellets by the phenol procedure contained very little radioactivity when compared to homogenates of intact pellets. This again suggests that the labeled material is not bound to RNA or DNA.

Subcellular Fractionation. Purified tritiated PHA from two separate lots (lots 4 and 5 of Table 1) of radioactive beans was used to test the subcellular localization of the tagged material by differential centrifugation of fragmented cells. Electron microscopic examination of the subcellular fractions indicated these to be relatively pure but with some interfractional contamination.

Table 3 shows the analysis of radioactivity of cell fractions of several cultures using two lots of 3HPHA.

The mitochondrial fraction consistently showed the highest activity, the nuclear fraction much less, and the least activity in the microsomal fraction. There was only a slight amount of radioactivity in the final supernatant from the different subcellular fractions.

Specific localization of the subcellular label by microscopy of autoradiographic preparations has been unsuccessful. Examination of smears of the different fractions showed a preponderance of labeled particles in the mitochondrial fraction. However, positive identification of the particles was not possible at such magnification. Electron microscopic localization has been unsuccessful due primarily to insufficient label being present in the thin sections to permit organelle localization with statistical reliability.

DISCUSSION

Attempts to label bean extracts by direct treatment with tritium gas with and without the use of catalysts resulted in loss of mitogenicity, indicating instability of the relatively large mucoprotein molecule. However, by growing beans in tritiated nutrient media, we were able, by column chromatography, to obtain a radioactive extract which yielded a relatively pure protein. Autoradiography of electrophoretic bands obtained from column fractions reflected increased purification and showed that the radioactive label was maintained during the processing. Greatly increased mitogenic activity of the purified protein was demonstrated in human leukocyte cultures. Electronic counting and sizing of cell nuclei proved a reasonably accurate and facile means of quantifying the degree of PHA-induced blastogenesis. The purified extract
had sufficient radioactivity to allow cellular localization by autoradiography and subcellular fractionation, but insufficient for precise organelle localization by electron microscopy.

The optimum concentration for mitogenic activity was 4µg./ml. of culture and if this concentration was exceeded, reduced mitogenic activity and increased toxicity were noted: if reduced, lowered mitogenic activity was noted. Some toxicity as evidence by morphological cellular changes was present at the optimum mitogenic concentration.

Observations of cell labeling in many serial autoradiographs suggest the following course of events. Within a few hours the ³HPHA caused labeling and agglutination of many leukocytes. Cellular breakdown liberated amorphous masses of labeled material. One wonders if liberation of such material might be conducive to blastogenesis since lymphocytes were often seen at the periphery of the masses and appeared to take up labeled material through their uropods (Fig. 4). Similar uptake by lymphocytes from adjacent labeled blast forms was noted later in culture. If such cellular uptake is involved in lymphocyte commitment it would tend to support the suggestion made by several investigators¹⁸,¹⁹ that there may be parallel (cell-to-cell) as well as vertical (cell division) commitment of lymphocytes.

It is noteworthy that although agglutination of red cells and leukocytes was common in these cultures, no label of red cells or surface label of leukocytes was noted at the times examined. As noted above, within a few hours of culture the label had already penetrated into the cytoplasm of the leukocytes. It is possible that only a fleeting contact of the PHA with cell membranes was necessary to alter the membrane sufficiently to bring about agglutination. If there are special membrane-surface reactive sites for antigenic action as has been proposed,²¹ such sites were not apparent with this labeling technique.

Studies of label distribution in blast cells indicated primarily cytoplasmic localization as evidenced by a considerably greater number of grains over the cytoplasm, compared with the number overlying the nucleus (Figs. 4–9). The label over the nucleus could probably be largely accounted for by label in the cytoplasm overlying the nucleus. Further evidence of preponderance of cytoplasmic localization was indicated by lack of DNA (chromosome) labeling, and on subcellular fractionation by the appearance of predominant radioactivity in the mitochondrial fraction. The radioactivity in the nuclear fraction on subcellular analysis may have been partly from interfractional contamination.

Association of the tritium label with the mitogenic molecule in the cells seemed likely for the following reasons: (a) Exposure of the bean plant to tritiated nutrient media should give a generalized labeling so that label of the mitogenic molecule would be expected; (b) The crude bean extract gave a generalized labeling of the blast cells, including the nucleus and DNA (chromosomes), whereas the purified PHA along with increased mitogenicity resulted in the label being confined primarily to the cytoplasm with much less nuclear labeling;* (c) cultures treated with residues from secondary peaks (which, though radioactive, were nonmitogenic) resulted in insignificant cell labeling; (d) cultures treated with a ten-fold dilution of the optimum dose of
the purified PHA produced numerous blast cells with distinct labeling, whereas
cultures treated with a similarly diluted unpurified PHA produced only a rare
labeled blast cell; and (e) most blast cells showed heavier label than untransformed lymphocytes after two days of culture.

The PHA did not appear to be closely associated with the RNA proteins
for several reasons: Lack of label in the nucleoli; the continued presence of
label in the cytoplasm of smears treated with perchloric acid; lack of label in
cell pellets extracted for RNA; and the fact that the microsomal fraction which
contains most of the RNA proteins was the least labeled of the subcellular
fractions. Lack of DNA label was indicated by lack of nuclear and chromo-
somal labeling and by lack of label in DNA extracts of cell pellets. It did not
seem likely that the mitogenic molecule had been significantly degraded into
smaller moieties or free tritium water since in that case one would have ex-
pected a more generalized labeling of the cell. Also, the supernatant of the
differential centrifugation fractions was relatively free of activity.

Though assay for radioactivity of subcellular fractions showed the mito-
ochondrial fraction to have the greatest activity, we were unable to achieve
specific visualization of label in individual mitochondria. It was noted, how-
ever, that cellular label was frequently concentrated in the cytoplasm near the
uropod (Fig. 9) which suggests association with mitochondria which are
known to be numerous in metabolically active regions.

Localization of activity in the mitochondrial fraction poses interesting spec-
ulations. Mitochondria have been found to be numerous in blast forms. Possibly PHA in some way activates the mitochondria. Increase in ATP, oxidative enzymes, and glycolysis have been reported to occur in these transforming cells. These metabolic events might promote the greatly in-
creased RNA synthesis which has been shown to occur in these cells prior to
DNA synthesis. Synthesis of DNA may later be stimulated by a feedback
mechanism.

Since it is generally believed that PHA is nonspecific in action compared
with specific antigens, perhaps PHA may bypass an initial step required by
these antigens in bringing about blastogenesis. Of course it is possible that it
may act by an entirely different mechanism that occurs with more specific
antigens.

SUMMARY

A tritiated bean extract was obtained from bean plants (Phaseolus vulgaris)
grown hydroponically in nutrient media containing various concentrations of
tritium water. Purification was accomplished by ammonium sulfate precipita-
tion and column chromatography using DEAE, CM-52 cellulose columns, and
finally by Sephadex G-100 gel filtration. The purified product showed greatly
increased mitogenic activity and electrophoretically showed a single labeled
protein band on electrophoresis. Radioactivity of the purified PHA varied
between 0.1–0.5 μCi/mg, which, though not as high as desirable, was sufficient
for autoradiographic and subcellular fractionation studies in human leukocyte
cultures. Within a few hours cytoplasmic localization of the label was noted
in most of the cells in culture, including neutrophils and larger mononuclear
cells. Breakdown of many of these cells during the first 24 hr. resulted in labeled amorphous basophilic staining masses of cell particles. Though agglutination of cells was commonly seen, the lack of label in red cells or surface label of leukocytes was notable. Blast forms which appeared by the second day showed prominent labeling which appeared to be largely cytoplasmic as evidenced by grain distribution. Several reasons were discussed which made it seem likely that the mitogenic molecule was represented in the cell label. Assay of subcellular fractions showed the major portion of radioactivity in the mitochondrial fraction. Specific activity, however, was too low to permit electron microscopic localization of label in individual organelles. Lack of label of RNA and DNA proteins was demonstrated by cellular distribution of the label and chemical extraction of RNA and DNA. Interesting speculation arises as to whether the PHA may stimulate mitochondrial activity and induce RNA synthesis and blastogenesis. Further experiments are in progress to obtain a purer PHA with higher specific radioactivity in order to more precisely define the site and mechanism of action of the mitogen.

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