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José Corcino, ... , Samuel Waxman, Victor Herbert

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Research Article

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In the present study, CMLL were incubated in Trisbuffered Hank's basal salt solution (pH 7.2) containing 0.1% human serum albumin to study release of B₁₂BP into the medium. B₁₂BP was released continuously and in increasing amounts over a 5 hr period at 37°C; this release was inhibited almost completely when the cells were incubated at 4°C and by about half as much in the presence of *N*-ethylmaleimide (1 mmole/liter). Cycloheximide (50 µg/ml) had no effect on the release of B₁₂BP but significantly inhibited incorporation of leucine-³H into leukocyte protein. G incubated with 20 mg/ml of compound 48/80, an experimental histamine-releasing agent, had a 6-fold increase in release of B₁₂BP over a 2 hr period.

Subcellular fractionation studies of human granulocytes demonstrate that most of the B₁₂BP is associated with the granular (20,000 g) layer with an excellent correlation observed between its subcellular distribution and that of acid phosphatase.

These findings suggest that the release of B₁₂BP from G is mediated by an active process and provide further evidence that granulocytes are secretory as well as phagocytic cells.

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Release of Vitamin B₁₂-Binding Protein by Human Leukocytes In Vitro

JOSÉ CORCINO, STEPHEN KRAUSS, SAMUEL WAXMAN, and
VICTOR HERBERT

From the Department of Medicine, Mount Sinai School of Medicine of the City University of New York, New York 10029, the Veterans Administration Hospital, Bronx, New York 10468, and the Department of Pathology, College of Physicians & Surgeons, Columbia University, New York, New York 10032

ABSTRACT Human granulocytes (G) contain a vitamin B₁₂-binding protein (B₁₂BP). There is evidence that chronic myelogenous leukemia leukocytes (CMLL) may synthesize B₁₂BP. Our prior studies suggested that intact, living intravascular G synthesize and release such protein into extracellular compartments in vivo.

In the present study, CMLL were incubated in Tris-buffered Hank's basal salt solution (pH 7.2) containing 0.1% human serum albumin to study release of B₁₂BP into the medium. B₁₂BP was released continuously and in increasing amounts over a 5 hr period at 37°C; this release was inhibited almost completely when the cells were incubated at 4°C and by about half as much in the presence of *N*-ethylmaleimide (1 mmole/liter). Cycloheximide (50 µg/ml) had no effect on the release of B₁₂BP but significantly inhibited incorporation of leucine-³H into leukocyte protein. G incubated with 20 mg/ml of compound 48/80, an experimental histamine-releasing agent, had a 6-fold increase in release of B₁₂BP over a 2 hr period.

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These findings suggest that the release of B₁₂BP from G is mediated by an active process and provide further evidence that granulocytes are secretory as well as phagocytic cells.

INTRODUCTION

It has been known for more than a decade (1) that the sera of patients with chronic myelogenous leukemia (CML) contain high levels of a vitamin B₁₂-binding pro-

tein with alpha-electrophoretic motility. This led various investigators (2-5) to study granulocytes in relation to their capacity to bind vitamin B₁₂ when added in vitro. The binding capacity for additional vitamin B₁₂ has been reported as 0.06-0.08 ng/10⁷ cells (3), 1.5 ng/10⁷ cells (4), and 8.9 ng/10⁷ cells (5).

In 1966, Simons and Weber (6) reported that human granulocytes in vitro are capable of synthesizing a protein with elution properties similar to that of the leukocyte vitamin B₁₂ binder. In recent studies from our laboratory,¹ we have found an excellent correlation between the serum unsaturated vitamin B₁₂-binding capacity and the total blood granulocyte pool. These studies supported the concept that at least part of the serum B₁₂-binding proteins derive from intact, intravascular granulocytes. The studies to be described were undertaken in an attempt to throw further light on this hypothesis.

Our observations support the concept that the leukocyte vitamin B₁₂-binding protein is actively secreted by granulocytes. Subcellular fractionation studies of human granulocytes demonstrate that most of this protein is associated with the granular layer.

METHODS

Gel filtration studies. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. A 2.5 × 85 cm column was packed as described by Flodin (7). The buffer used was 0.075 M Tris, containing 0.075 M phosphate, 1 M NaCl, and 0.02% Na azide.

Granulocytes obtained from a patient with CML by the dextran sedimentation-hypotonic shock method (8) were suspended in phosphate buffer (0.2 mole/liter, pH 7.2) and frozen-thawed three times. The resulting suspension was centrifuged at 5000 rpm for 10 min and the supernatant removed. To 0.2 ml of the supernate, 900 pg of ⁵⁷CoB₁₂

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(specific activity 200 $\mu\text{Ci}/\mu\text{g}$; Amersham-Searle) was added, followed by a 30 min incubation at room temperature. 2 ml of normal serum was then added and the sample applied to the column. 3-ml fractions were collected in a Buchler fraction collector (Buchler Instruments, Inc., Fort Lee, N. J.). Radioactivity in each tube was determined in an automated gamma well counter; optical density was measured in a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Release and incorporation studies. Heparinized blood obtained from a patient with both CML and untreated pernicious anemia² was centrifuged in a refrigerated centrifuge at 3000 rpm for 4 min. The plasma was removed and the cells were washed three times with Tris-buffered Hank's balanced salt solution (TH) (Microbiological Associates, Inc., Bethesda, Md.), pH 7.2. The cells were then resuspended in Tris-buffered Hank's containing 0.1% normal (not salt-poor) human serum albumin (THA) (Cutter Laboratories, Berkeley, Calif.).

Aliquots of the cell suspension were added to 10-ml vacutainer tubes followed by the appropriate inhibitor or stimulator. In the experiments carried out at 37° or 4°C, the tubes were equilibrated to the desired temperature before adding the cell suspension. The average nucleated cell count in the final suspension was 50,000 cells/mm³. After incubation, the tubes were centrifuged at 3000 rpm for 4 min in a refrigerated centrifuge. The supernatants were then carefully removed with a Pasteur pipette.

The vitamin B₁₂-binding capacity (B₁₂BP) was determined in aliquots of the supernates by coated charcoal assay (9). In that procedure an excess of ⁵⁷CoB₁₂ (specific activity 10 $\mu\text{Ci}/\mu\text{g}$; E. R. Squibb & Sons, New York) was added to each fraction and the tubes incubated at room temperature for 30 min. The excess vitamin B₁₂ was removed by the addition of 2 ml of a hemoglobin-coated charcoal suspension and the radioactivity in the supernates was measured in an automatic Picker gamma counter (Picker X-Ray Corp., Cleveland, Ohio). Results were expressed as net counts per minute of ⁵⁷CoB₁₂ in supernates.

Leucine-³H with a specific activity of 5.2 Ci/mmoles per liter was obtained from Tracerlab Div., Richmond, Calif. In the experiments where release of radioactive protein into the media was measured, the cell suspension was preincubated with 2 $\mu\text{Ci}/\text{ml}$ of packed cells of leucine-³H for 1 hr at 37°C, the cells were washed twice with TH, and the cell suspension was resuspended in THA. At different time intervals, the tubes were centrifuged at 3000 rpm for 4 min in a refrigerated centrifuge and the supernates were removed carefully with a Pasteur pipette. 0.2 ml of 50 mg/ml of bovine serum albumin (Armour Industrial Chemical Co., Chicago, Ill.) was added to aliquots of the supernates followed by 1 ml of 50% trichloroacetic acid (TCA). The tubes were left overnight at 4°C and the next morning the precipitate was washed twice with 3 ml of 10% TCA and dissolved in 1.0 ml of Hyamine (Rohm and Haas Co., Philadelphia, Pa.). After adding 15 ml of scintillation fluid (10), the vials were counted in a Picker Liquimat 330 liquid scintillation counter. Results were expressed as net counts per minute of leucine-³H.

² At the time the blood was obtained, this 43 yr old Negro male (C. S.) had a serum B₁₂ level of 448 pg/ml, despite the fact that his only treatment for pernicious anemia was 5 μg of B₁₂ intramuscularly 1 month earlier. We are following him in an attempt to determine whether his B₁₂ deficiency is keeping his CML from worsening.

In the studies with iododeoxyuridine-¹²⁵I, 0.2 ml of 50 mg/ml of bovine serum albumin was also added to the supernates followed by precipitation with TCA. Precipitate was then counted in a gamma counter.

When incorporation of leucine-³H by leukocytes was studied, the red cells in the suspension were removed by hypotonic shock lysis and the resultant white cell button was precipitated with 10% TCA at 4°C. The precipitate was washed twice with 10% TCA and then dissolved in 1 ml of Hyamine. 15 ml of scintillation fluid was then added and the vials were counted in a liquid scintillation counter. Results were expressed as net counts per minute of leucine-³H. In the studies with iododeoxyuridine-¹²⁵I, the cell buttons were counted after hypotonic shock lysis and TCA precipitation.

Leukocyte viability was ascertained by the Trypan blue exclusion method.

Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, Mo.), *N*-ethylmaleimide from Calbiochem (Los Angeles, Calif.), and Trypan blue from Fisher Scientific Company (Pittsburgh, Pa.). The compound 48/80 was supplied by Dr. S. W. Singleton of Burroughs Wellcome & Co., Tuckahoe, N. Y., as a dry powder. Final concentrations of 50 $\mu\text{g}/\text{ml}$, 1 mmole/liter, and 20 mg/ml were used for cycloheximide, *N*-ethylmaleimide, and compound 48/80, respectively. All compounds were dissolved in TH.

Subcellular fractionation studies. Leukocytes for subcellular fractionations were obtained by dextran sedimentation of heparinized blood obtained from patients with various myeloproliferative disorders, followed by hypotonic shock lysis. The cell button obtained was suspended in 0.34 M sucrose to which a few drops of heparin (10,000 U/ml) were added and passed several times through a microsyringe filter holder (Millipore Corp., Bedford, Mass.) until adequate rupture of cells was obtained as determined by phase contrast microscopy. The fractions were designated as follows: *nuclear and particulate matter*, sedimented at 2000 g; *granular*, sedimented at 20,000 g; *ribosomal*, sedimented at 78,000 g (afterwards fractionated in sucrose density gradients into an upper and lower component); and the *supernate* representing the supernatant of the 78,000 g spin. Electron microscopic and further enzymatic characterization of the different fractions will be published separately by Krauss.³

Acid phosphatase in the subcellular fractions was determined by the method of Valentine and Beck (11) as modified by Hirschhorn, Hirschhorn, and Weissman (12), utilizing beta-glycerophosphate as substrate.

RESULTS

Gel filtration studies. Gel filtration on Sephadex G-200 (Fig. 1) of the vitamin B₁₂-binding protein in leukocytes (solid line) demonstrates a homogeneous peak which eluted with an approximate molecular weight of 115,000. The interrupted line represents the optical density at 280 m μ of normal serum, in the same column.

Release of vitamin B₁₂-binding protein (B₁₂BP) by human leukemic leukocytes. As shown in Fig. 2, B₁₂BP is released continuously and in increasing amounts at 37°C, but not at 4°C, over the 5 hr period studied. Addition of compound 48/80, at a concentration of 20 mg/

³ Krauss, S. The synthesis of glycoproteins by human granulocytes. Submitted for publication.

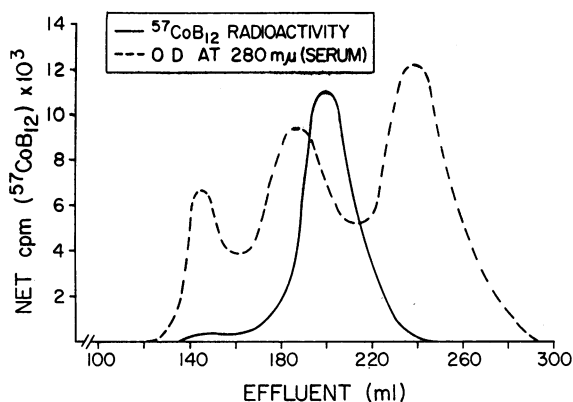


FIGURE 1 Sephadex G-200 chromatography of vitamin B₁₂ leukocyte-binding protein. Solid line represents ⁵⁷CoB₁₂ radioactivity expressed in net cpm × 10³. Interrupted line represents optical density at 280 mμ of serum run through the same column.

ml, results in a marked increase in release. It should be pointed out that approximately 20% of the granulocytes became Trypan blue positive at the end of 1 hr after treatment with this concentration of 48/80. Cycloheximide, at concentrations of 50 μg/ml, showed no effect upon the release of B₁₂BP. *N*-ethylmaleimide (1 mmole/liter) resulted in 60% inhibition of release. Granulocytes remained Trypan blue negative at the concentrations of both cycloheximide and *N*-ethylmaleimide utilized in our studies.

Fig. 3 defines further the effect of compound 48/80 upon release of B₁₂BP. As shown, the effect of 48/80 is quite rapid, reaching a plateau during the first 30 min. The effect of 48/80 is markedly temperature dependent and is not inhibited by a 15 min preincubation with *N*-ethylmaleimide.

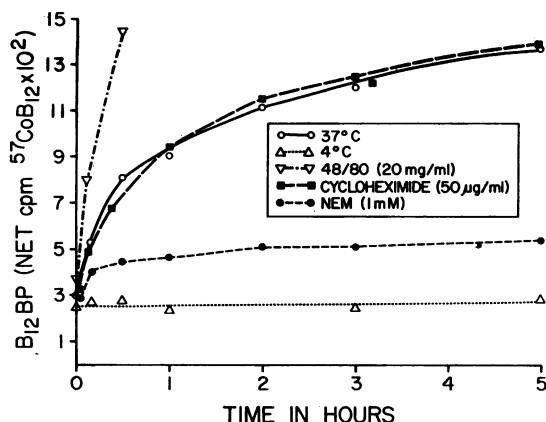


FIGURE 2 Release of B₁₂BP by human leukemic leukocytes in vitro: ○—○ at 37°C; △···△ at 4°C; ▽---▽ in the presence of 20 mg/ml of compound 48/80, at 37°C; ■---■ in the presence of 50 μg/ml of cycloheximide; ●---● in the presence of 1 mM *N*-ethylmaleimide.

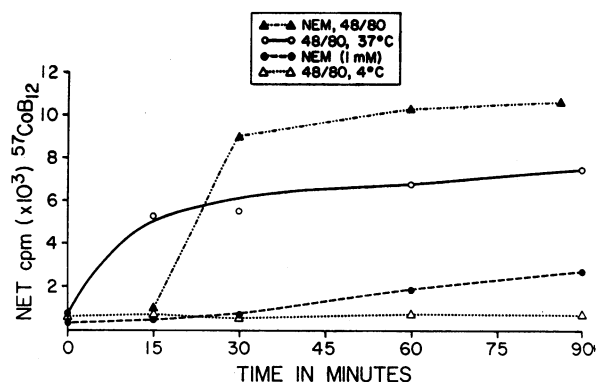


FIGURE 3 Release of B₁₂BP by human leukemic leukocytes in vitro: ▲···▲ preincubated with 1 mM *N*-ethylmaleimide for 15 min and then exposed to 20 mg/ml of compound 48/80; ○—○ 20 mg/ml of 48/80 at 37°C; ●---● 1 mM *N*-ethylmaleimide; △···△ 20 mg/ml of 48/80 at 4°C.

Incorporation of leucine-³H by human leukemic leukocytes. As can be seen in Fig. 4, the incorporation of leucine-³H is continuous over a 5 hr period, reaching a plateau at approximately 1 hr. Practically no incorporation is seen when the cells are incubated at 4°C or in the presence of *N*-ethylmaleimide and/or cycloheximide.

Radioactive protein released from intact leukocytes. Fig. 5 depicts the release of radioactive protein by leukocytes over a 2 hr period. Release is continuous over this period of time at 37°C, but not at 4°C. Compound 48/80 results in a marked enhancement of release, this latter not being affected by cycloheximide. *N*-ethylmaleimide suppresses release by approximately 50%.

Incorporation and release into the medium of iododeoxyuridine-¹²⁵I. Fig. 6 shows the increasing incorporation of iododeoxyuridine-¹²⁵I into leukocytes over a 5 hr period. As can be seen, very little acid insoluble radioactivity is released into the medium over the study period.

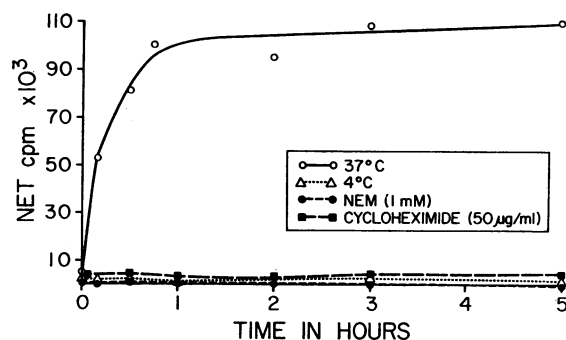


FIGURE 4 Incorporation of leucine-³H by human leukemic leukocytes: ○—○ at 37°C; △···△ at 4°C; ●---● in the presence of 1 mM *N*-ethylmaleimide; ■---■ in the presence of 50 μg/ml of cycloheximide.

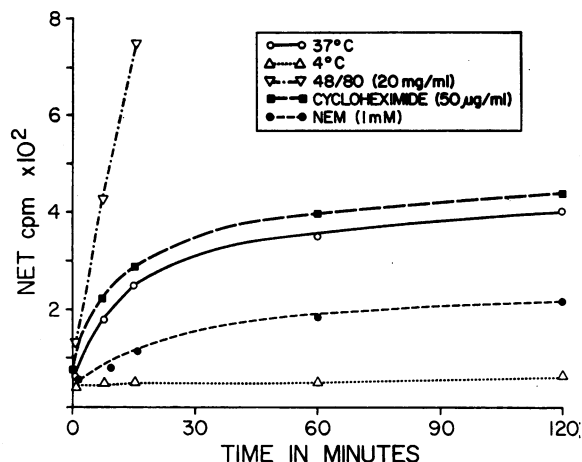


FIGURE 5 Radioactive protein (acid insoluble radioactivity in the supernates) released from intact leukocytes preincubated with leucine- ^3H : \bigcirc — \bigcirc at 37°C ; \triangle — \triangle at 4°C ; ∇ — ∇ in the presence of 20 mg/ml of 48/80; \blacksquare — \blacksquare in the presence of 50 $\mu\text{g}/\text{ml}$ of cycloheximide; \bullet — \bullet in the presence of 1 mM *N*-ethylmaleimide.

Subcellular localization of vitamin B₁₂-binding protein. Fig. 7 shows the subcellular distribution of the leukocyte B₁₂BP. As depicted, most of the B₁₂BP is present in the 20,000 g or granular fraction of leukocytes.

Fig. 8 presents a comparison of the distribution of the B₁₂BP with that of acid phosphatase in the various subcellular fractions. As can be seen, most of the activity of both substances is concentrated in the 20,000 g or granular layer.

DISCUSSION

Sonicates of human granulocytes are capable of binding from 4 to 9 ng of vitamin B₁₂ per 10^7 cells when added in vitro. When such sonicates are submitted to gel filtration on Sephadex G-200 (Fig. 1), the granulocyte-binding protein, marked with $^{57}\text{CoB}_{12}$, elutes as a single, homogeneous peak with an approximate

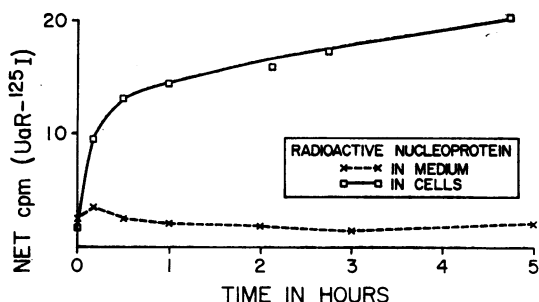


FIGURE 6 Incorporation and release into the medium of iododeoxyuridine- ^{125}I (UaR- ^{125}I). Radioactive nucleoprotein acid insoluble radioactivity): X---X in medium; \square — \square in cells.

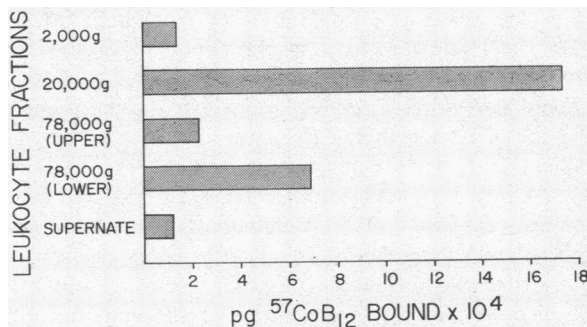


FIGURE 7 Subcellular localization of B₁₂BP in human leukocytes. Expressed as total pg of $^{57}\text{CoB}_{12} \times 10^4$ bound by each fraction.

molecular weight of 115,000. Because of similarities between the granulocyte vitamin B₁₂-binding protein and one of the vitamin B₁₂ binders present in human serum (13, 14), it has been suggested that granulocytes are the source of this serum protein (5). This concept is further supported by the presence of high levels of this protein in the sera of patients with CML (1) and the fact that granulocytes in vitro synthesize a protein with elution properties similar to the leukocyte B₁₂ binder (5).

Recent studies¹ from our laboratory have demonstrated a better correlation between the serum unsaturated vitamin B₁₂-binding capacity (UB₁₂BC) and the total blood granulocyte pool, than that observed between the UB₁₂BC and the granulocyte turnover rate. These and the current studies support the concept that at least part of the serum B₁₂-binding proteins are actively secreted by intact, intravascular granulocytes rather than being just part of the debris from destroyed granulocytes.

In the current studies, in vitro release of the leukocyte vitamin B₁₂ binder proved to be temperature dependent (released at 37°C but not at 4°C) and inhibited by *N*-ethylmaleimide, a sulfhydryl inhibitor known to inhibit the release of beta-glucuronidase (induced by calcium in the leukocidin-treated rabbit granulocyte) (15). The exact mechanism by which this action of *N*-ethylmaleimide is mediated is unknown; it has been suggested that protein extrusion does not occur because

FRACTION	% OF TOTAL B ₁₂ BP	% OF TOTAL ACID PHOSPHATASE
2,000g	5	5
20,000g	60	64
78,000g (UPPER)	8	9
78,000g (LOWER)	24	15
SUPERNATE	4	7

FIGURE 8 Comparison of distribution of B₁₂BP with that of acid phosphatase in subcellular fractions of human leukocytes. Expressed as per cent of total in each fraction.

contact between the granule and cell membrane can no longer be made due to a change of state in the cell cytoplasm (15).

Compound 48/80, a polymeric condensation product of *p*-methoxyphenethylmethylamine and formaldehyde, previously shown to enhance the release of histamine by mast cells (16) and granulocytes (17), also increases the release of B₁₂BP. This stimulation is temperature dependent and, interestingly, is not affected by preincubation with *n*-ethylmaleimide (Fig. 3). Since 20% of the cells become Trypan blue positive at the end of 1 hr, when exposed to the concentration of 48/80 used in our experiments, it is possible the effect of this compound upon granulocytes, at this concentration, is toxic.

Cycloheximide, a known inhibitor of protein synthesis, suppresses incorporation of leucine-³H by leukemic leukocytes almost completely (Fig. 4). At the same concentration it exerts no effect upon the release of B₁₂BP (Fig. 2) or upon the release of acid insoluble radioactivity by leukocytes prelabeled with leucine-³H (Fig. 5). Similar findings have been recently reported (18) for the release of amylase by parotid gland slices in vitro.

Since the release of B₁₂BP or acid insoluble radioactivity in our system could be a reflection of cell disruption rather than or in addition to an active secretory process, studies were undertaken using labeled iododeoxyuridine as a precursor of leukocyte nucleoproteins. Incorporation of the precursor was continuous at 37°C over a 5 hr period, but there was no significant increase in the labeled nucleoprotein (acid insoluble radioactivity) present in the media (Fig. 6). These findings provide evidence against significant cell disruption, and support active secretion of B₁₂BP from storage depots within the cell (as opposed to secretion of continuously produced B₁₂BP).

A variety of substances (phagocytin, hyaluronidase, lysozyme, ribonuclease, deoxyribonuclease, beta-glucuronidase, histamine) have been shown to be actively secreted by leukocytes in vitro (19), in response to different stimuli. These compounds have been shown by subcellular fractionation studies to originate from granules within the leukocytes. Our studies demonstrate that the B₁₂BP is also predominantly found in the granular layer of granulocytes (Fig. 7), with an excellent correlation observed between its subcellular distribution and that of acid phosphatase (Fig. 8). The presence of significant B₁₂BP (and acid phosphatase) in the lower layers of the 78,000 g button could represent either contamination of this fraction with the granular layer or heterogeneity of the granules resulting in different sedimentation velocities. Such heterogeneity has been recently described from several laboratories (20-22). Should such prove correct, then we would expect that the 78,000 g button will prove to contain more granules containing B₁₂BP than granules containing acid phosphatase.

Whether the B₁₂BP is an enzyme (13) or one of the proteins related to specific types of granules (21) remains undetermined.

Thus, the current studies provide further evidence that granulocytes are secretory as well as phagocytic cells. This would help explain the apparent correlation of B₁₂BP with total body granulocyte pool in hypo- and hyperleukocytic states (23, 24). A preliminary report of some of these studies has recently been presented (25).

ACKNOWLEDGMENTS

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