The proximal scolopale cells lie in the blood-space of the tibia and are attached at their distal extremities to the accessory cells. The latter are closely bound together by a network of tendrils, presumably collagenous in nature, which come together in a broad stem to attach to the cuticle.

Depending on the conditions of damping, the sudden application of a force to a mass will initiate a harmonic free vibration of that mass. The displacement, \( x \), is given by an equation of the form

\[
x = Ae^{-\alpha t} (\cos \omega t - \gamma)
\]

Where \( \omega \) is the angular velocity at the natural frequency, and \( A \) and \( \gamma \) are constants proportional to the applied force. Under normal conditions this vibration will soon be damped out. If the applied force is itself harmonic, as in the cases considered here, it follows there will be an initial transient vibration set up. This will revert to the vibration due to the impressed force as soon as the free vibration has been damped out. The displacement will then be given by an equation of the form

\[
x = X \cos \omega t
\]

where \( X \) is the maximum displacement, and \( \omega \) is the angular velocity of the impressed force. Therefore the initial effect of the forced vibration of the leg will be to set the cells of the subgenual organ into free (transient) vibration, but since the distal cells are bound to each other and to the cuticle, their natural frequency will be different from that of the proximal cells. Hence while the transient vibrations last, there will be a rapid and complex variation of forces at the junctions of the proximal and distal cells, which could cause the nervous discharge. If this is so, any sufficiently abrupt displacement from equilibrium or steady-state conditions must be expected to initiate nervous discharge, which is clearly in accord with the results obtained.


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An Alkali Resistant Factor with B12 Activity for Protozoa and Man1

Our studies on the effect of flushing doses of cyano-cobalamin (vitamin B12) in dogs and man revealed that B12 displaced not only hepatic bound B12 but also an alkali-resistant thermofactor (ARF) which supported the growth of the B12-requiring microorganisms Lactobacillus leichmannii, Escherichia coli 113-3, Euglena gracilis and Ochromonas malhamensis. Growth could not be attributed to deoxyribo-sides since the latter two organisms do not respond to nucleic acid derivatives. Also, ARF failed to support growth of a thymine-thymidine requiring E. coli mutant. Unlike B12 or related cobamides, ARF is stable at 118°-121°C and pH 11.5-12 for 30-60 min without added reducing agents. ARF was not detected in the circulation of man unless displaced by B12. It was found in human and beef liver and also in alkali extracts from the culture medium of a thermophilic bacillus grown at 55°C. The present communication describes methods of preparation and some of the chromato-
graphic, microbiological, and clinical properties of ARF.

Materials and Methods. Source of ARF. (a) Human: Hepatic venous blood was obtained from 12 normal subjects and 8 patients with cirrhosis 5 min to 2 h after intravenous administration of 100 μg of B12. Surgical liver biopsies were obtained from 8 normal subjects and percutaneous biopsies from 2 patients with pernicious anemia. (b) Animal: Available commercial beef liver powders were analyzed for their ARF content. The best source was liver extract concentrate 1:20 obtained from Nutritional Biochemicals Corp., Cleveland (Ohio). (c) Microbial: Bacillus coagulans ATCC 12990, a thermophile produced ARF. It was grown at 55°C in a medium consisting of corn steep liquor (5.0 ml), NaCN (2.0 mg), cobalt (4.0 mg as CoSO4·7H2O), citric acid·H2O (100 mg), triethanolamine (300 mg), distilled water (to 100 ml) adjusted to pH 6.1 with KOH. Cobalt was not essential for ARF production, the same concentration of iron, added as FeSO4·7H2O could be substituted. Although ARF activity appeared without these ions, either ion stimulated ARF production by the bacillus. The medium was inoculated with an homogenate from a loopful of B. coagulans grown on nutrient agar overnight. Maintenance and growth of these thermophiles have been described. ARF assay. B12 and ARF were assayed with (a) L. leichmannii ATCC 7830, (b) the mutant E. coli 113-3, (c) E. coli 113-3, (d) E. coli 1309 (1958).

1 These studies were supported by NIH Grants A-1497 (C1); C-4956 (C2); H-4590 (C2): and Clinical Research Center Grant OG-171-R1; and a Grant from the National Vitamin Foundation.
8 A. W. Johnson and A. Todd, Vitamins and Hormones 15, 1 (1957).
10 C. M. Leevy, Practical Diagnosis and Treatment of Liver Disease (P. B. Hoeber, New York 1957).
gracilis strain Z and (d) *O. malhamensis* by previously described methods.  
(a) Serum: Serum was obtained from blood allowed to clot at room temperature for 3–4 h. One ml of serum was diluted with 4 ml of acetic acid buffer i.e. 0.5% trans-aconitic acid, adjusted to pH 4.5 with KOH. The solution was autoclaved at 16 psi, 118–121°C for 30 min. The coagulum was removed by centrifugation and the supernatant adjusted from pH 4.5 to 11.5–12 with KOH and reautoclaved for 30 min. After autoclaving the solution was then readjusted from 11.5–12 to pH 7.0 with HCl and assayed for ARF. Controls to evaluate B$_{12}$ destruction consisted of duplicate sera to which 0.1 µg/ml of crystal-line B$_{12}$ was added.

(b) Liver: 25 g of beef liver was dissolved in 500 ml of acetic acid buffer and treated as described for serum. Assay of the initial B$_{12}$ content of the liver powder was determined after acid hydrolysis. 5 mg of B$_{12}$ was added to 1 g of liver powder to evaluate the effects of alkaline destruction. After readjustment of the pH to 7.0 with HCl the liver solution was lyophilized to a dry powder; 0.1 mg% of this powder was used for estimating ARF activity. Unless readjusted the pH was static after all procedures. Human liver biopsies were processed in the same manner.

(c) Microbial: After overnight incubation at 55°C, the *B. coagulans* culture medium was adjusted to pH 4.5 with citric acid and autoclaved. The debris was removed by filtration and the supernatant adjusted to pH 11.5–12 with KOH and treated as described for serum. A 1:5000 dilution of the supernatant was used for assay. This dilution of crude preparations was found desirable to diminish the toxic effects of contaminating salts on the growth of the assay organisms.

**Bioautography.** 0.1 ml of test solution obtained from hepatic venous serum, liver (1.0 mg per ml) and microbial sources was chromatographed in a descending system at room temperature on Whatman No. 1 paper strips 52 cm. The solvent system was n-butanol:acetic acid:water (4:1:5). After chromatography the paper was placed on a B$_{12}$ assay medium (Baltimore Biological Co., Baltimore, Md.) to which 10 mg per ml of agar was added. It was then seeded with a culture of *L. leichmannii*: 0.2 µg per ml of 2,3,5-tetrazolium red served as a marker for differentiating growth. Bioautography with *L. leichmannii* differentiated B$_{12}$ from deoxyribosides in the substances being assayed.

**Results.** Activity of ARF derived from various sources is given in the Table. The ARF activity is given in B$_{12}$.

<table>
<thead>
<tr>
<th>Source</th>
<th>Organism</th>
<th><em>Ochromonas malhamensis</em> (µg/ml)</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hepatic vein serum</td>
<td>B$_{12}$</td>
<td>ARF</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>Addition of 100 µg of B$_{12}$/ml of serum</td>
<td>104 000</td>
<td>0</td>
<td>26 900</td>
</tr>
<tr>
<td>30 min after B$_{12}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Microbial</td>
<td><em>Bacillus coagulans</em> ATCC 12900</td>
<td>5 900</td>
<td>4800</td>
</tr>
<tr>
<td>3. Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver biopsy</td>
<td>1 769</td>
<td>233</td>
<td></td>
</tr>
<tr>
<td>Biopsy from pernicious anemia</td>
<td>45</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Commercially prepared beef liver</td>
<td>2 400</td>
<td>1300</td>
<td></td>
</tr>
</tbody>
</table>

Weights since B$_{12}$ was used as a growth standard. The growth-promoting activity of ARF and B$_{12}$ was approximately the same for the four B$_{12}$-requiring microorganisms. Assay results with *O. malhamensis* are presented because this organism is the best parameter of B$_{12}$ activity. Normal serum contained no ARF: ARF was only present in hepatic vein serum after giving B$_{12}$; its activity was highest 5–30 min after intravenous B$_{12}$ (Table). Naturally occurring B$_{12}$ and B$_{12}$ added to extracts of serum, beef liver and fermentation broth of *B. coagulans* were destroyed by the alkaline treatment. Normal human liver and beef liver contains ARF; no ARF was present in liver biopsy specimens obtained from 2 patients with pernicious anemia in relapse. The richest source of ARF was the bacterial fermentation broth of *B. coagulans*. Apparently, this thermophile produces only ARF and not B$_{12}$ since alkaline hydrolysis results in little loss of activity.

**Chromatographic and bioautographic studies** showed that B$_{12}$ subjected to acid hydrolysis elicited two growth areas, 8 and 18 cm from the origin. No growth was seen after alkaline hydrolysis. Addition of B$_{12}$ to normal serum did not alter these results. ARF from each of the sources produced an area of growth at the origin and a lighter growth zone 5 cm from the origin. ARF obtained from liver also exhibited some activity 45 cm from the origin, attributed to the presence of thymidine in liver extracts. There was no deoxyribose activity in alkaline extracts of serum or thermophiles.

The hemopoietic activity of ARF was evaluated in 2 patients with pernicious anemia in relapse who exhibited a macrocytic anemia, megaloblastic bone marrow, achlor-hydra, a normal serum folic acid and a serum B$_{12}$ of 21 and 23 µg/ml (normal 200–1000 µg/ml). Both patients were maintained on a folic acid and B$_{12}$ deficient diet, and on this regimen exhibited a reticulocyte count of 0.1–0.5% without significant changes in the hematocrit. A total of 0.84 µg or *O. malhamensis* responsive ARF was given intramuscularly to the first patient. Its administration was followed by an increase of reticulocytes from 0.5 to 12%, a rise in hematocrit from 22 to 37%, and a reversal of the megaloblastic bone marrow to one of normal morphology. At the time of the maximum hemopoietic response the serum B$_{12}$ level had decreased from 25 to 4 µg/ml and hepatic tissue content of B$_{12}$ was reduced from 45 to 20 µg/mg of dried liver (normal 1000–3000 µg/mg). 1 µg of *O. malhamensis* activity ARF given orally on two occasions produced a similar response in a second patient with pernicious anemia. Subsequent Schilling tests were compatible with a diagnosis of pernicious anemia in both patients. Further details of these clinical studies will be recorded elsewhere.

**Stability to alkaline hydrolysis** differentiates ARF from crystalline B$_{12}$, coenzyme B$_{12}$, hydroxycobalamin, folic acid or folinic acid, since these substances are completely destroyed by autoclaving at pH 11.5–12 for 30 min. The chemical and microbiological properties of ARF also differentiate it from erythropoietin, a