

alfalfa meal or bovine feces (2). It was shown to be stable to heat, acid, alkali, and drying and was not a known B vitamin, amino acid, peptide, purine, pyrimidine, oleic acid, or mineral, although some of these substances are undoubtedly also required. Hungate (3) concluded that the factors required for growth of this organism are not all present in the feed consumed by the animal but are in part produced by the accompanying microorganisms. The purpose of the present work was to determine the nature of this unknown factor.

The assay procedure involved the use of an anaerobic culture method previously described (1). The basal culture medium contained minerals, B vitamins, purines, pyrimidines, casein hydrolyzate (enzymatic), a carbonic acid-bicarbonate buffer system, and either glucose or cellulose (0.3 percent Whatman No. 1 filter paper) as carbon source. Growth in mediums containing cellulose was estimated by the visible loss of cellulose from the tube and growth in glucose mediums was estimated by amount of turbidity after about 18 to 20 hr of incubation using the Cenco-Sheard Spectrophotometer set at 420 m μ .

The factor was found in the ether extract of rumen fluid extracted at pH 2 but not in the residue. The reverse was true on ether extraction at pH 10. The factor was found in the volatile acid fraction on acid steam distillation and was not volatile when distilled under alkaline conditions. Fractionation of the volatile acids by chromatographic technique (4) showed most of the activity to be present in the valeric acid fraction.

The assay of commercially available volatile fatty acids showed that none of the acids, C₂ through C₆ and their isomers, n-heptanoic, or n-caprylic, allowed a significant amount of growth when added singly to the basal medium. However, when all combinations of two of the acids isobutyric, n-valeric, isovaleric, DL- α -methyl-n-butyric, or n-caproic were assayed, good growth occurred if one was a straight-chain acid and the other, a branched-chain acid. This observation was confirmed by assaying all possible combinations of three of these acids. In this case the only combination not allowing growth was that containing the three branched-chain acids.

Studies to date show that the branched-chain component can be any one of the acids isobutyric, isovaleric, or DL- α -methyl-n-butyric. Isocaproic, trimethyl acetic, or the straight-chain acids C₂ to C₈ will not replace these acids. The straight-chain component can be any one of the acids C₅ to C₈. Acetic, propionic, n-butyric, or lauric acid will not replace these acids. A small amount of activity for the straight-chain component was found in palmitic and stearic acid.

Minimum concentrations for good growth when n-valeric and isovaleric acids were used were about 3 and 1.5 μ M, respectively, per 10 ml of medium.

It seems probable that the acids chiefly concerned with the growth response due to rumen fluid are isobutyric, isovaleric, and DL- α -methyl-n-butyric acid for the branched-chain component, and n-valeric and

n-caproic acid for the straight-chain component. These have all been found in significant amounts in rumen fluid (5, 6). The probable origin of these acids by Stickland reactions from amino acids has been discussed by El-Shazly (7).

Details of this work will be reported elsewhere.

Note added in proof: After this work was submitted for publication, O. G. Bentley *et al.* reported that the volatile fatty acids, n-valeric, isovaleric, isobutyric, and n-caproic, or their amino acid precursors stimulated cellulose digestion and the conversion of urea nitrogen into protein by rumen microorganisms as measured by the "artificial rumen" technique (8).

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In vitro Differentiation between Auto- and Isoimmune Antibodies by Protamine and Trypsin

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It has been shown that low concentrations of heparin inhibit the agglutination of red cells coated with auto-antibodies (1). This phenomenon was believed to be either competitive, similar to the mechanism of hemagglutinating virus antagonists (2), or due to the strongly negative charge of heparin. Although auto-antibodies were selectively inhibited, no interaction with isoantibodies was observed (contrary to virus antagonists). The effect of a positively charged colloid on these hemagglutinins was therefore investigated (3). Protamine sulfate (Lilly) in concentrations from 0.01 percent (pH 6.11) to 1.0 percent (pH 5.98) was used.

It was found that saline suspensions of normal cells, cells sensitized with anti-D (albumin) typing serum (4), and cells from patients with idiopathic acquired hemolytic anemia were agglutinated by protamine sulfate. Normal, trypsinized cells (5) were not affected by any of the concentrations used. Control series showed that acidification alone (6) will not cause agglutination in the absence of protamine, nor will neutralization of a protamine solution (pH 7.1) affect its agglutinating ability. The effect of cell fragility was also negated, since the resistance to hypotonic saline was the same before and after trypsinization. The possibility of protamine inactivation by minute amounts of trypsin adsorbed on the cell surfaces (7)

is unlikely. Such traces, if present, would exhibit tryptic activity also upon incubation with unprocessed x-ray film. Control experiments showed that this was not the case.

In an attempt to prevent the agglutination by protamine, red cells were suspended in 0.5 to 2.0 percent saline dilutions of bovine albumin. Differences were observed between the dilutions necessary to protect normal cells, cells sensitized with anti-D antibody, or coated with autoantibodies (patients' cells). Similar results were noted with saline suspensions of cells exposed to varying concentrations of protamine sulfate. The critical values varied with cells from different individuals. However, normal cells were always the most resistant, and Rh-sensitized cells were the most susceptible to agglutination (Table 1).

Table 1. Effect of mixtures in promoting (+) or inhibiting (–) the agglutination of test cells. Red blood cell suspensions, 2 percent. Volume of reactants, 0.1 ml each. Titer of direct Coombs' test on patients' cells and sensitized cells, 1:256. Cell suspensions incubated with the mixtures for 10 min at 37°C and subsequently centrifuged for 1 min at 1000 rev/min.

Agglutinating medium	Normal cells	Patients' cells (auto-antibodies)	Sensitized cells (isonatibodies)	Trypsinized cells
Protamine 1% Saline	++++	++++	++++	–
Protamine 0.08% Saline	–	+	++++	–
Protamine 1% Albumin 2%	–	–	++++	–
Protamine 1% Albumin 0.5%	–	++	++++	–
Protamine 1% Trypsin 1%	–	–	++++	–
As above, pre-incubated 10 min	–	–	++++	–
Trypsin 1% Saline	–	–	++++	–
Saline alone (Control No. 1)	–	–	–	–
2% albumin alone (Control No. 2)	–	–	–	–

When trypsin and protamine were added simultaneously to the cell suspensions, only cells coated with anti-D antibody agglutinated. Incubation of protamine and trypsin for 10 min prior to their addition to these cell suspensions had the same effect. Since trypsin digests protamine, it became apparent that the enzyme itself must function as the agglutinating agent. Tests performed with trypsin alone confirmed its selective ability to agglutinate cells coated with anti-D antibodies (Table 1).

This effect is independent of the concentration of trypsin from 0.05 to 1.0 percent but requires incuba-

tion and seems to diminish with decreasing direct Coombs' titers.

These observations substantiate the reported differences between auto- and isoimmune antibodies (1). The trypsin effect also suggests a connection between two seemingly unrelated phenomena: (i) trypsinized cells are more susceptible than normal cells to agglutination by gamma globulin (8); (ii) the isoantibodies in hemolytic anemia of the newborn are gamma globulins, whereas the autoantibodies in acquired hemolytic anemia, are not (9).

References and Notes

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3. This work was supported by a grant from the Albert Einstein Medical Center, Philadelphia, Pa.
4. 5.0 ml of a 2-percent O/CDE cell suspension in saline incubated for 30 min with 0.015 ml of anti-D (albumin) typing serum gave a direct Coombs' test titer of 1:256.
5. 1-percent saline solution of "Difco 1:250" trypsin plus 1/10 its volume phosphate buffer pH 7.1; filtered before use. Trypsinization of cells was achieved by incubating a normal cell suspension with 1/10 volume enzyme mixture for 10 min.
6. Cell suspensions made up in a 0.02M NaCl-phosphate buffer, pH 6.0.
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On the Sources of Soil Phosphorus Absorbed by Mycorrhizal Pines

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Previous studies have indicated limited absorption of phosphorus by nonmycorrhizal pines in certain soils (1, 2). Either successful mycorrhizal inoculation or heavy applications of inorganic phosphates were effective in overcoming acute phosphorus deficiency (2). The mode of action of the mycorrhizal association is unknown. Among hypotheses are the possibility that activity of the extensive mycelium ramifying from the root tips might release phosphorus from the soil organic matter in the immediate vicinity of unuberized root tips (1, 3) or the phosphorus might be transmitted to the root through fungal hyphae (4). Release into the soil solution remote from the root tips is unlikely, since benefit to pine was not shared by a companion grass (2).

Distinction between soil inorganic and organic phosphorus forms is possible. When inorganic P³² is added to the soil, it rapidly undergoes dilution and isotopic exchange with the inorganic soil phosphorus in accessible sites (5). Phosphorus in organic forms, being held by covalent bonds, remains unlabeled except as P³² is gradually incorporated by microbial synthesis (6). Accordingly, in a soil so treated, a plant able to utilize organic forms would be characterized by a