animals the full supplement at a time most conducive to overcoming the acute phase of the deficiency.

The present data indicate that vit. $B_{12}$ is about equally effective by oral or injection administration in promoting growth of young rats on highly specialized depletion diets. The deficiency of vit. $B_{12}$ induced in rats by these rather extreme dietary means does not, therefore, appear to evoke a failure in utilization of the vitamin, but rather a true deficiency and enhanced need for the vitamin itself.

The following additions to the diet failed to yield a comparable growth response, and even appeared to have an inhibitory effect: 5-10% purified casein, 5-10% of various primary-grown and brewer's yeasts, and additional folic acid. The addition of thymidine, 25-50 mg per kg diet, betaine hydrochloride 0.5%, or additional choline 0.1% likewise did not stimulate growth in absence of vit. $B_{12}$.

The growth promoting action of vit. $B_{12}$ in rations containing iodinated protein has recently been reviewed.[10] Betheil and Lardy[11] have further compared the effectiveness of vit. $B_{12}$, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid rats. The ration used by these authors was also based on purified casein, but did not contain a sulfa drug. It is worthy of note that mortality is high among our control animals and that death is generally preceded by appearance of hemorrhage about the nose and paws. Vit. $B_{12}$ clearly prevents this syndrome and has a marked effect toward longevity under the dietary conditions imposed.

The question of the equivalence of vit. $B_{12}$ with U.S.P. anti-pernicious anemia units can only be answered by assays in human pernicious anemia patients. Microbiological and animal assays may serve as a useful guide, however, toward this goal. The data presented provide some evidence that concentrated liver extracts which show a correlation by microbiological assay of 1 U.S.P. unit approximately equal to 1 $\mu$g of vit. $B_{12}$, show a similar approximate equivalence by rat assay.

*Summary.* The growth response of vit. $B_{12}$ depleted rats under the conditions studied is proportional to the vit. $B_{12}$ administered in the critical range of .025-.1 $\mu$g per rat day. Oral and injection administration of the vitamin at critical levels yield approximately equal growth responses. Addition of the vitamin to the diet, multiple dosing or single dosing the first day of assay also appear roughly equivalent. A preliminary correlation between the results of microbiological and rat assays as applied to injectable liver extracts was obtained.

Received August 4, 1949. P.S.E.B.M., 1949, **72**.

[10] *Nutrition Reviews*, 1949, **7**, 183.

[11] Betheil, J. J., and Lardy, H. A., *J. Nutrition*, 1949, **37**, 495.

## Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins from Perchloric Acid Filtrates of Plasma.* (17347)

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It has been shown[1] that the "proteose" of human plasma is a mixture of mucoproteins with isoelectric points which are low in comparison with those of the principal plasma proteins. The present communication deals in more detail with the electrophoretic behav-

* The electrophoresis studies were supported by a grant from the United States Public Health Service and the isolation studies were supported by the American Cancer Society. Contribution No. 224 from the Department of Biochemistry and Nutrition, University of Southern California, School of Medicine.

[1] Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.

ior of this material.

*Methods. Preparation of materials.* The mucoprotein fractions were isolated in the manner already described from perchloric acid filtrates of pooled, normal, human plasma, and in one case (Preparation No. 44) from the plasma of a patient with gastric carcinoma whose plasma mucoprotein-tyrosine level was 4.9 mg % in comparison to normal levels of 2-4 mg %.[2] We are indebted to Dr. M. P. Petermann of the Memorial Hospital of New York City for the lyophilized sample of this plasma. Preparations were thoroughly dialyzed after isolation, and were lyophilized.

*Chemical characterization.* The lyophilized samples were analyzed for carbohydrate, glucosamine, nitrogen, and tyrosine† by the methods previously described.[1]

*Electrophoresis.* Samples were prepared for electrophoresis by dissolving them in the desired buffer, with subsequent dialysis against two portions of buffer for a period of at least 24 hours in each portion of buffer. The temperature was 5°C during the dialysis.

In most cases a small amount of insoluble material was present, and solutions were centrifuged immediately before electrophoresis. In order to cover a wide range of pH values with each preparation, it was necessary to recover material from the electrophoresis cell and use it in subsequent measurements. Recovered solutions were concentrated by placing them in a dialysis bag and blowing air over the bag at room temperature. This procedure, and the limited amounts of material available, made it impractical to control the concentrations, so that there was considerable variation in protein concentration in different experiments. It also seems probable that some change in composition took place during repeated recovery and concentration. This has contributed to the difficulty of identifying components at different pH values in some instances.

The buffer systems were prepared with diethyl barbiturate, acetate, citrate or phosphate. All buffers were made to give a final concentration of 0.08 M NaCl and 0.02 M with respect to the monosodium salt of the buffer system or the disodium salt in the case of phosphate. HCl was added to obtain the desired pH, so that the ionic strength was 0.1 in each case except at pH 6, where disodium phosphate was employed In this case the ionic strength was correspondingly greater.

Electrophoresis was carried out in an 11 ml Tiselius cell at 2.0°C, with a potential gradient of between 5 and 6 volts per cm. The conductivities used to calculate the potential gradient were also determined at 2°C on the buffer used to fill the electrophoresis cell. The pH of the buffer, also determined after dialysis, was obtained with a Beckman pH meter at room temperature.

The boundaries were photographed, generally after 3 hours, using the Philpot optical system,[3] and at a magnification of 1.05 to 1. The photographs were enlarged somewhat over 2 times for making the required measurements.

*Experimental results.* The preparations used in this study had the chemical characteristics shown in Table I. These preparations show the characteristically high carbohydrate and glucosamine content previously reported.

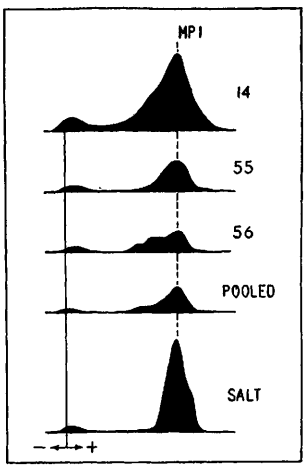


FIG. 1.

Electrophoresis patterns of mucoprotein preparations at pH 8.4. Descending boundaries. Preparations No. 14, No. 55, No. 56, and "pooled," are from perchloric acid filtrates of pooled, normal plasma. The "Salt" preparation was prepared entirely by ammonium sulfate fractionation, and contains 31% albumin.

2 Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 617.

† Although reported as tyrosine, it is recognized that the Folin's phenol reagent used is not specific for this amino acid.

3 Philpot, J. S. L., *Nature*, 1938, **141**, 283.

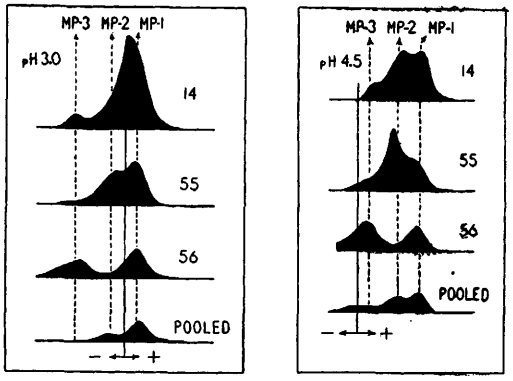


FIG. 2.
Electrophoresis patterns of mucoprotein preparations at pH 3 and 4.5. Boundaries descending toward the anode.

The electrophoresis patterns of the descending boundaries obtained on some of the preparations from human plasma are reproduced in Fig. 1 and 2. In the patterns of Fig. 1, obtained at pH 8.4, is included the pattern of a preparation made entirely by ammonium sulfate fractionation without preliminary deproteinization with perchloric acid. Although this preparation contains 31% albumin, it does demonstrate the greater degree of homogeneity which can be obtained with respect to the major mucoprotein component (MP-1) by such a fractionation procedure.‡ In Fig. 2, obtained at pH 3.0 and 4.5, are indicated components which we have designated as MP-1, MP-2, and MP-3. At pH 6 and higher, the resolution into well-defined components is less satisfactory and we have only indicated the position of MP-1 in Fig. 1.

In Fig. 3 we have attempted to present the pH-mobility curves for the descending boundaries of these components. Although we feel reasonably well satisfied with the general characteristics in the region below a pH of about 5.5, the uncertainty regarding MP-2 and MP-3 at higher pH's has been indicated in the figure.

*Discussion.* Human plasma mucoprotein prepared by the preliminary removal of other plasma proteins with perchloric acid may contain at least 3 electrophoretically different components, and the spreading of the boundaries suggests that the degree of heterogeneity is even greater than this. The proportions of the components vary considerably from preparation to preparation, and despite the rather drastic initial treatment with perchloric acid the preparations may be somewhat modified when solutions are repeatedly recovered by evaporation at room temperature. Because of these limitations, conclusions must be drawn with some caution. The preparations labeled No. 14, No. 55, and "pooled" are the most typical of preparations which we have presented for discussion. The analytical values which are given in Table I are in reasonably good agreement for these three. Preparation No. 56 had the least typical electrophoretic behavior and had the lowest values for glucosamine, carbohydrate, and tyrosine, except for the material prepared exclusively by salt fractionation, which contained 31 per cent albumin.

The one component which is seen in all preparations, including the "salt" preparation is MP-1. This material has a mobility of $-6.4 \times 10^{-5}$ cm$^2$ sec$^{-1}$ volt$^{-1}$ in the descending boundary at pH 8.4, and would appear in the $\alpha_1$-globulin fraction of serum at this pH. This component is still strongly negatively charged at pH 4.5, and is isoelectric at a pH of

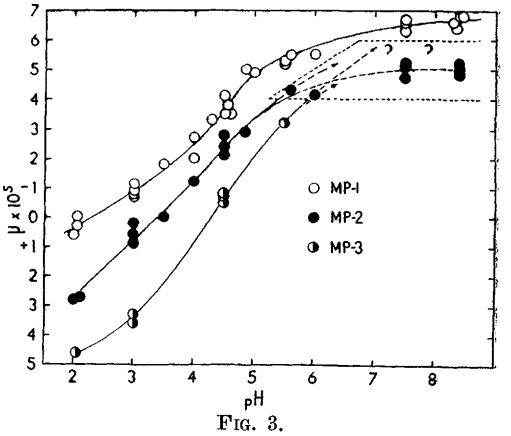


FIG. 3.
Mobility of mucoprotein components as a function of pH. Identification of components other than MP-1 is uncertain above pH 6, and this area of uncertainty is indicated by enclosing it in the dotted lines. Principally because of the spread of peaks, mobilities at all pH values are subject to considerable error.

‡ This preparation was made by Mr. Henry Weimer, and results of further refinements of this method will be reported in the near future.

TABLE I.

| Preparation | Source | Yield mg/100 ml plasma | Nitrogen, % | Glucosamine, % | Carbohydrate, % | Tyrosine, % |
|---|---|---|---|---|---|---|
| No. 14 | Human, normal | 38 | 8.1 | 10.1 | 15.6 | 3.9 |
| No. 44 | " cancer | 100 | 7.5 | 8.9 | 16.3 | 3.9 |
| No. 55 | Human, normal | 28 | 9.0 | 9.1 | 15.7 | 4.3 |
| No. 56 | " " | 32 | 7.1 | 8.1 | 13.6 | 3.4 |
| Pooled* | " " | — | 8.3 | 9.8 | 14.3 | 3.6 |
| Salt fract.† | " " | 72 | 7.25 | 7.6 | 13.2 | 3.1 |

* Five separately isolated lots were pooled for this sample.
† Contained 31% albumin by electrophoresis, assuming equal refractive increments per g of albumin and MP-1.

about 2.3 in citrate buffer.

At pH 8.4, some material slower than MP-1 is always seen, and at pH 4.5 there is generally a considerable amount of material with a mobility of about $-2.3 \times 10^{-5}$. This material which we have designated MP-2, has an isoelectric point at about pH 3.4. From the analytical data, it must also be a mucoprotein.

The material designated as MP-3 might conceivably be contaminated with albumin. The mobility is not very different from that of albumin in this buffer, and the broad peaks for MP-3 would generally overlap the mobility for albumin at pH 4.5. Small amounts of albumin might also contribute to the broadening of the electrophoretic peaks at pH 8.4. However, preparation No. 56 could not contain enough albumin to account for the peak observed at pH 4.5. Pending the isolation of more homogeneous preparations, the status of this component must remain in considerable doubt. The isoelectric point, however, is in the region of pH 4.3.

It is clear from the study of these preparations that mucoprotein components MP-1 and MP-2 should be demonstrable by electrophoresis of plasma at a suitable, more acid pH than is generally employed. Such a study has been made by Petermann *et al.*,[4] and further studies in this direction are being presented in paper IV of this series.[5]

The sample isolated from a patient with cancer (No. 44) presented essentially the same characteristics on electrophoresis as did the pooled sample. The similarity with respect to proportion of components was greater than the analytical data would have led one to suspect. This case, at least, would indicate that the increased amount of mucoprotein in cancer represents an increase in normal components rather than the appearance of an abnormal component.

Although the groups responsible for the low isoelectric points of these mucoproteins have not been identified, it seems likely that they are at least in part sulfuric acid ester groups. Sulfur in excess of that accounted for by cystine and methionine has been observed in preparations of mucoprotein from rat blood,[6] horse serum,[7] and human plasma.[1]

*Summary.* The electrophoretic behavior of mucoproteins isolated from perchloric acid filtrates of human plasma has been studied over the pH range of 2 to 8.4. There are at least 3 electrophoretic components in such preparations, the isoelectric points being approximately 2.3, 3.4 and 4.3. The major component has the lowest isoelectric point and travels with a mobility characteristic of $\alpha_1$-globulin at pH 8.4. The increase in the mucoprotein level isolated from the plasma of a patient with gastric cancer was largely in this acid mucoprotein fraction.

4 Petermann, M. P., Karnovsky, D. A., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

5 Mehl, J. W., Golden, F., and Winzler, R. J., PROC. SOC. EXP. BIOL. AND MED., 1949, **72**, 110.

6 Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.*, 1944, **4**, 417.

7 Mayer, K., *Z. f. Physiol. Chem.*, 1942, **275**, 16.

Received August 4, 1949. P.S.E.B.M., 1949, **72**.