PREPARATION AND PROPERTIES OF PURIFIED INTRINSIC FACTOR

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Received May 4, 1960

The recent isolation of cobamide coenzymes (Barker et al. 1958; Weissbach et al. 1959) has stimulated interest in the role of these coenzymes in several metabolic reactions. The use of intrinsic factor (IF), the substance necessary for the oral utilization of vitamin B_{12}, has been found to be helpful in studying the mechanism of these reactions (Barker, 1960; Ochoa, 1960). There have been brief reports in the literature on the partial purification of IF, which is thought to be a mucoprotein, (Wijmenga, 1957; Jacob et al., 1959), but the properties and the precise data on the potency of these preparations have not been previously reported. The present report is concerned with the preparation and properties of highly purified IF, fully active at 0.3 mg, the most potent reported to date. The purified IF is not a mucoprotein.

Preparation of IF - Minced hog pyloric mucosa was extracted twice with 4 parts v/w of 2% NaCl at 10-12°C. The combined extracts, obtained by filtering through cheesecloth, were adjusted to pH 9.0 with 1 N NaOH. After 30 minutes at pH 9.0, the solution was adjusted to pH 1.5 - 2.0 with 1 N HCl. The precipitate that formed was removed by centrifugation. The resulting supernatant solution was adjusted to pH 4.5 with 1 N NaOH and 194 gm of $(NH_4)_2SO_4$ per liter of supernatant solution was added. After 4 hours, the mixture was centrifuged, the precipitate discarded, and 286 gm of $(NH_4)_2SO_4$ per liter added to the resulting supernatant solution. After standing overnight at 10-12°C the mixture was centrifuged and the supernatant was dialyzed to remove the $(NH_4)_2SO_4$. Some in-

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soluble material formed on dialysis and was removed by centrifugation. The resulting supernatant, termed AS fraction, was fully active at 5 mg by the modified Schilling urinary excretion test. (Ellenbogen and Williams, 1958).

Further fractionation was carried out using DEAE - cellulose. (Peterson and Sober, 1956). Ten grams of AS fraction was dissolved in 0.025 M NaH_2PO_4 buffer, pH 7.5 and placed on a 4.5 x 50 cm column. Elution was carried out at 4°C with the same buffer. A large protein peak which came off the column first was always inactive at test levels up to 2 mg. IF activity appeared at the end of the descending slope of the first peak and continued partly through the middle section of the curve. This material was active at 1 mg. A final protein band which appeared at the end of the column was eluted with pH 5.5 phosphate - 0.2 M NaCl buffer. This material was devoid of activity at a level of 2 mg.

The active portions from the columns can be further purified by rechromatography which yields a well defined protein band. This can be more readily done by ultrafiltration through a parlodion membrane at 4°C. The ultrafiltration serves both to further purify IF and remove buffer salts from the protein solution. The activity was retained in the ultrafilter (ultrafiltrate residue) and this material was active at 0.3 mg by the modified Schilling test and the fecal excretion test (Heinle, et al, 1952). The results of these tests are shown in Table I. A summary of the purification is shown in Table II.

Properties of Purified IF - Examination of the purified material in the ultracentrifuge revealed that the preparation was still heterogeneous. Immunochemical studies by the Ouchterlony double diffusion technique revealed at least 3 relatively weak bands with antisera prepared by immunizing rabbits with the AS fraction.

The purified IF contained only 5% blood group A substance as compared to 20% group A substance (as determined by hemagglutination inhibition tests) in the AS fraction. The hexosamine content (Belcher et al, 1954) of the purified material was less than 2% whereas the cruder AS fraction contained 7.3% hexosamine. The IF activity was therefore separated from the bulk of the mucoprotein. According to the classification proposed by Meyer (1953) the purified preparation

Table I

ACTIVITY OF ULTRAFILTRATE RESIDUE FRACTION - (LEVEL - 0.3 mg)

Prep. No.	Without IF	With Preparation	With Standard IF
	Radioactive Vitamin B_{12} Excreted in 48 Hours (μg.)		
Urinary Excretion Test			
732	0.01	0.29	0.32
820	0.01	0.23	0.22
821	0.01	0.31	0.21
826	0.01	0.18	0.22
834	0	0.25	0.33
Fecal Excretion Test			
	Radioactive Vitamin B_{12} Absorbed (μg.)		
700	0.18	0.57	0.55
732	0.23	0.55	0.60

Table II

SUMMARY OF PURIFICATION OF HOG INTRINSIC FACTOR

Step	Protein g.	Activity ** mg.	Total Units of Activity	Yield %
Hog gastrointestinal tissue	400	60	6666	-
Extract	140	30	4666	70
$(NH_4)_2SO_4$ fraction (AS)	10	5	2000	30
DEAE-cellulose	0.680*	1	680	10.2
Ultrafiltrate residue	0.189	0.3	576	8.7

* Estimated on the basis of optical density measurement.
** Level at which the urinary excretion test gave excretion values comparable to standard IF (Ellenbogen and Williams, 1958)

would not be classified as a mucoid or a mucoprotein. The purified IF contained 12% nitrogen and was devoid of peptic or tryptic activity. Heating IF, active at 1 mg., in a boiling water bath for 15 minutes or direct boiling of the IF for 2 minutes results in about 50% destruction of activity. This partial inactiva-

tion is not prevented by heating 1 mg. of the IF together with 2 µg. of vitamin B_{12}. The vitamin B_{12} binding capacity of this highly active preparation was 5 µg per mg. of IF and was considerably higher than preparations of lower potency. This lends support to the suggestion that the mechanism of vitamin B_{12} absorption is related to its binding to IF. (Wijmenga, 1957).

Acknowledgments - We wish to thank Dr. E. A. Kabat, Columbia University for performing the tests for blood group A activity, Dr. D. L. Mollin, Post - graduate Medical School, London, England for the fecal excretion assays, Mrs. C. Gussoni, Lederle Laboratories who performed most of the immunochemical studies, and E. Marier and W. Mirtschink, Lederle Laboratories for technical assistance.

References

Barker, H. A., Weissbach, H., and Smyth, R. D., Proc. Nat. Acad. Sci. U. S., 44, 1093 (1958).

Barker, H. A., Personal Communication (1960).

Belcher, R., Nutten, A. J., and Sanbrook, C. M., Analyst, 79, 201 (1954)

Ellenbogen, L. and Williams, W. L., Blood, 13, 582 (1958)

Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C. and Prusoff, W. H. Trans. Assn. Am. Phys., 65, 214 (1952)

Jacob, T. A., Williams, D. E., Howe, E. E. and Glass, G. B. J., Arch Biochem. Biophys., 81, 522 (1959)

Meyer, K., in W. H. Cole (Editor), Some Conjugated Proteins. Rutgers Univ. Press, New Jersey, p. 64 (1953).

Ochoa, S., Personal Communication (1960).

Peterson, E. A. and Sober, H. A., J. Am. Chem. Soc., 78, 75 (1956)

Weissbach, H., Toohey, J., and Barker, H. A., Proc. Nat. Acad. Sci. U. S., 45, 521 (1959)

Wijmenga, H. G., in H. C. Heinrich (Editor), Vitamin B_{12} and Intrinsic Factor, Ferdinand Enke Verlag, Stuttgart, p. 156 (1957).