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Biochemical Studies of Bacterial Derivatives. X. Preparation of Human Tubercle Bacillus Protein MA-100.*

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(Introduced by F. M. Huntoon.)

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In an effort to produce a potent tuberculin in a concentrated form and with the least possible chemical manipulation, Seibert[1] resorted to ultrafiltration, a method based on the conclusion from previous experiments that the active principle in tuberculin which is responsible for eliciting skin reactions in tuberculous guinea pigs is a water-soluble protein of the nature of an albumin. While this appears to be a promising method for concentrating the protein without incurring the possibility of denaturation, the end product is still complex since it retains all of the non-dialyzable constituent of the original filtrate among which are metaproteins, proteoses, and a large proportion of carbohydrate.

The aim of this investigation was to determine (1) whether the carbohydrate is linked up with the protein molecule in the form of a glucoprotein, and (2) if not, to prepare a protein fraction free from carbohydrate in order that its biological activity would not be masked by its presence. A method is outlined below for preparing such a fraction, to which we have given the designation of Human Tubercle Bacillus Protein MA-100 to conform with Johnson's scheme of nomenclature.

A culture of Tubercle bacilli (H-37) was grown on Long's synthetic medium and then filtered through a filter candle. To 11,000 cc. of this filtrate was added 7150 gm. $(NH_4)_2SO_4$. The precipitated protein soon rose to the top of the vessel and after standing several hours it was separated from the liquid by filtering through a Büchner funnel. The precipitate was purified by 8 successive reprecipitations. The precipitate from the last operation was dissolved in 1000 cc. distilled water, filtered through paper, and to the clear solution was added 30 cc. of Rimington's[2] salt buffer mixture (pH 4.7). Five volumes of 95% ethyl alcohol were added to the solution. A flocculent greyish-white precipitate formed, which gradually settled.

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* This investigation was done in cooperation with the Medical Research Committee of the National Tuberculosis Association.

1 Seibert, F. B., *J. Biol. Chem.*, 1928, lxxviii, 345.

2 Rimington, Claude, *Biochem. J.*, 1929, xxiii, 428.

After standing over night the clear supernatant liquid was siphoned off, the precipitate thrown on a Büchner funnel and sucked dry. The precipitate which was readily removed from the paper was suspended in 320 cc. of distilled water, and just enough NaOH solution added to bring it to a pH of 7.4. The slight haziness of the liquid was removed by filtering through paper.

The clear yellow solution was adjusted to pH 4.7. A heavy flocculent precipitate formed which was allowed to settle. After standing several hours, the almost clear supernatant was siphoned off, and the precipitate was centrifuged. The precipitate was purified in this manner by three consecutive isoelectric precipitations. The final precipitate was dissolved in 320 cc. distilled water with the aid of a few drops of N/2 NaOH. Twenty cc. of this solution were reserved for test purposes. The remainder was reprecipitated once more at its isoelectric point, the precipitate was removed by centrifuging and then hydrolyzed with $Ba(OH)_2$ according to Rimington's[2] method in order to see whether any carbohydrate might be split off.

The supernatant from the fourth isoelectric precipitate gave only a weak Molisch test. Likewise one cc. of the purified Tubercle bacillus protein gave a faint positive Molisch. The purification process had removed almost completely the carbohydrate. The reducing properties of the carbohydrates in the various fractions were determined before and after hydrolysis by the Shaffer-Hartmann micro method.

TABLE I.
Distribution of Carbohydrate in Various Fractions Determined as Reducing Valued in Terms of Dextrose.

	Before Hydrolysis		After Hydrolysis	
	% Dextrose	Gm. Dextrose	% Dextrose	Gm. Dextrose
Original filtrate 11,000 cc.	0.144	15.8	0.485	53.3
8th ammonium sulphate precipitate dissolved in 1000 cc. water	0.005	0.05	0.390	3.9
Supernatants from 3 isoelectric precipitates made up to 1000 cc.	0.0000	0.0000	0.240	2.4
4th isoelectric ppt. hydrolyzed by the Rimington method—volume 94 cc.	0.17	0.016	0.028	0.026

An analysis of the data above shows, therefore, that 99.95% of the total reducing substances in the original filtrate was removed by this process. The carbohydrate, therefore, is not present in the form of a glucoprotein. The trace still present in the protein is probably occluded and is not an integral part of the protein molecule.

The portion of the purified protein set aside for test purpose assayed 12.6 mgm. protein per cc. and gave a positive Biuret, Xanthoproteic, Millon and Molisch but a negative sulphur and Hopkins-Cole test. It contained a small amount of phosphorus. The protein solution was diluted and tested on tuberculous guinea-pigs and humans. It gave a positive typical skin reaction in doses as low as 0.00005 mgm. on humans. In comparison with O.T. and tuberculin made by Seibert's ultrafiltration method, this protein was relatively less toxic.

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The Relation of Vitamin D to Deposition of Calcium in Bone.*

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Quantitative studies in our laboratory[1] have shown that when the food of the growing animal (rat) includes a liberal allowance of vitamin D and a constant supply of phosphorus (0.42%), the calcium content of the bodies as determined at intervals during the period of growth is markedly influenced by the calcium content of the food, as varied in these experiments from 0.16 to 0.50% of calcium in the dry food mixture.

In the present experiments we find further, that in rats receiving a basal diet containing a generous allowance of calcium and phosphorus in good proportions (calcium, 0.74%; phosphorus, 0.58%) but with the vitamin D supply restricted practically to a bodily store acquired by 21 to 28 days of age, the growing body has at a given age about the same calcium content as has been acquired by similar animals which received 0.32% calcium, 0.42% phosphorus, and a liberal supply of vitamin D.

The calcium content of the femurs of rats which had been kept from the age of 21 or 28 days to the age of 56 days on the vitamin-D-deficient diet which we have previously described[2] is about twice as high as in otherwise similar rats fed the high-calcium rickets-producing diet of Steenbock (No. 2965) for the same length of time.

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[1] Booher, L. E., Dissertation, Columbia University, 1928.

[2] Sherman, H. C., and Stiebeling, H. K., *J. Biol. Chem.*, 1929, lxxxiii, 497.