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Beta- and Gamma-Globulin Tetanus Antitoxin of the Hyperimmune Horse

PAST work has indicated that fractionation of diphtheria antitoxic horse serum yields fractions of which the dilution ratio, and hence the firmness of combination, may show considerable variations. There was a definite correlation between the *in vitro/in vivo* ratio and the dilution ratio¹. Kekwick and Record² examined electrophoretically pure gamma- and beta-globulin from hyperimmune horse sera, and found that both fractions had antitoxic activity and that they differed in their $L+L_f$ ratios and in their speed of flocculation. It was of interest to investigate whether this was a general phenomenon, or one confined to the diphtheria toxin-antitoxin system, by studying the relation between tetanus toxin and horse tetanus antitoxic sera.

Qualitative differences between tetanus antitoxic sera have been observed by Hartley (quoted in ref. 3) and Petrie⁴. Dilution tests⁵ on two sera and two fractions from one of them indicated a similarity to the diphtheria toxin-antitoxin system, and Kekwick⁶ demonstrated that both gamma- and beta-globulin were antitoxic.

Gamma- and beta-globulin were obtained by fractionation with sodium sulphate followed by electrophoretic separation^{2,6} from sera of horses hyperimmunized intramuscularly with tetanus toxoid. Flocculation always gave multiple zones. The flocculation zone due to tetanus toxin was determined by preparing floccules near each of the indicating mixtures, using proportions providing excess of toxin or antitoxin. The floccules were washed and then added to a suitably diluted solution of antitoxin. Toxin-antitoxin floccules absorb considerable quantities of antitoxin and may be distinguished in this way from floccules derived from flocculation zones involving other antigens⁷. L_f and K_f were determined at 49° C. in phosphate buffer, $I = 0.2$, pH 8.

The extent to which toxin-antitoxin dissociates on dilution was tested in two ways: (1) the amount of an antitoxin was found that would neutralize an

$L+5$ dose of toxin and was compared with the amount required to neutralize an $L+250$ dose of toxin⁵; (2) the amounts required to neutralize the test dose of toxin in the mouse and in the guinea pig were compared. (M/G is ratio of L +units per ml. in the two cases.⁴)

It may be concluded that, in flocculating, tetanus-toxin-antitoxin systems resemble diphtheria toxin-antitoxin mixtures. The gamma-globulin combines more avidly than the beta-globulin with the toxin; it dissociates less on dilution.

This work will be presented in detail elsewhere. Our thanks are due to Dr. R. A. Kekwick for suggesting this investigation and for his encouragement.

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Connective Tissue Permeability and the Mode of Action of Hyaluronidase

THE following observations would seem to throw fresh light upon the question of the mode of action of hyaluronidase on connective tissue permeability.

A sheet of fresh fascia from the flank of a mouse was fixed as an occluding membrane across the end of a glass tube of some 0.5 cm. internal diameter: rubber rings were found to be the best method of sealing the membrane to the tube. Particular care was taken not to damage the membrane during dissection or to stretch it unduly afterwards.

Fascial membranes prepared in this way were always found to be permeable to physiological saline, and at a pressure of 12 cm. water the rate of flow through them was of the order of 60 μ l. per min. This rate was almost immediately increased by some ten times when hyaluronidase (Benger's 'Hylase', 10 units per ml.) was added to the saline perfusate. The same result was obtained with a saline extract of mouse testis.

This effect of hyaluronidase was counteracted by dilute solutions of certain substances of large molecular size. Thus when the following substances, at the w/v per cent concentration indicated, were perfused, the original state of permeability was restored within an hour: starch, 0.005 per cent; agar, 0.01 per cent; gum arabic and citrus pectin, 0.05 per cent; leaf gelatine, 0.1 per cent; chondroitin sulphate and dried serum, 0.2 per cent. Mouse serum at a dilution of 1/40 and human synovial fluid at 1/100 were similarly effective.

In the case of synovial fluid, and to some extent with serum and pectin, the slowing of flow was partly due to viscosity, since it took place rapidly and normal flow was restored almost at once when saline was substituted. But with starch, agar and gum arabic the slowing was gradual, regular and also permanent, inasmuch as no quickening of flow took place when saline was again perfused.

Antitoxin	K_f	L +units per ml. L_f		$\frac{M}{G} \times 100$	$\frac{L+250}{L+5} \times 500$
		a	b		
'Keeble' serum	145'(1)	1.5	2.2	87	68
'Keeble' gamma-globulin	69'(2)	2.6	4.4	60	55
'Keeble' beta-globulin	165'(1)	1.2	1.3	106	95

(1) 6 units/ml. of flocculation mixture.

(2) 4.2 units/ml. of flocculation mixture.

(a) Determined from $L+5$ measurements.

(b) Determined from $L+250$ measurements.

Connective tissue membrane perfused at a pressure of 12 cm. water. Acceleration of flow caused by hyaluronidase counteracted by dilute starch. Flow afterwards accelerated by ptyalin

With the exception of synovial fluid, where a slight effect was observed, hyaluronidase had no further accelerating action upon the slowed rate of flow brought about by perfusion with these substances. But it was found that ptyalin (human saliva diluted by 1,000) had an effect upon a starch-treated membrane which imitated the former action of hyaluronidase precisely (see graph).

It is apparent that certain substances of large molecular size can, even in considerable dilution, slow the rate of flow when passed through a fascial membrane. This effect is independent of viscosity, and in the case of starch was clearly due to the accumulation within the membrane of starch molecules or molecular aggregates. There is no reason to doubt that the same mechanism was mainly responsible for the slowing action of the other substances mentioned with the exception of synovial fluid, with which viscosity effects predominated.

Connective tissue may be looked upon as a fabric of protein fibres¹, and it would seem that some degree of 'water-proofing' of the fabric by large molecules is possible. On this view, the sudden rheological effect of hyaluronidase, an effect, one would think, far too rapid to indicate the action of this enzyme on any very great quantity of substrate, is due to removal of those hyaluronic acid molecules which normally, to some extent, 'waterproof' the fabric. It is significant that, after treatment with hyaluronidase, the normal permeability of the tissue could be quickly restored by concentrations of serum far smaller than found in most pathological exudates. In spite of its dramatic effects on fresh tissue, it would seem, therefore, that hyaluronidase must be of negligible importance in relation to the spread of such exudates.

A detailed account of these findings will be published in due course. I wish to thank Dr. C. R. Boyes, of Messrs. Allen and Hanburys, Ltd., for providing me with citrus pectin and chondroitin sulphate, and Dr. Frank Fletcher, of Benger's, Ltd., for a supply of 'Hylase'.

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Phosphorylation and Absorption of Sugars in the Rat

It is known that the absorption of sugar from the intestine is accompanied by an increased phosphorylation of sugar in the mucosa¹⁻³; but the evidence available does not show clearly if this phosphorylation plays a direct or an indirect part in absorption. It was felt that an examination of the properties of the hexokinase of the mucosa might help in deciding this question.

Homogenates of rat intestinal mucosa (1 gm. of tissue in 4 ml. homogenate) were employed, hexokinase activity being followed by the disappearance of free sugar from the digest in the presence of adenosinetriphosphate, using the method of Nelson⁴. The digest was incubated at 30° C. in open test tubes, and contained veronal-acetate buffer pH 8.0, 0.05 M; magnesium chloride, 0.01 M; sodium fluoride 0.06 M; as well as appropriate concentrations of sugar and adenosinetriphosphate. Total volumes of 1.5-2.5 ml. were used, of which one-third by volume was homogenate. No sugar disappearance occurred in the absence of adenosinetriphosphate. High concentrations of adenosinetriphosphate were required, because of the intense adenosinetriphosphatase activity of the homogenate. The phosphatase activity present did not interfere markedly with the sugar disappearance during the first 10 min. of the hexokinase reaction.

Water homogenates were used for studying the phosphorylation of D-glucose, D-fructose, D-mannose and D (-) xylose. At a sugar concentration of 0.0055 M, and an adenosinetriphosphate concentration of 0.033 M, the average hexokinase activity towards glucose was 0.80 ± 0.12 mgm. glucose disappearing per millilitre homogenate in 5 min. (15 rats). Water homogenates gave no activity towards D-galactose, as was found by Meyerhof with brain⁵; but, as with brain, good activity was obtained when Meyerhof's magnesium Ringer solution was employed in the preparation of the homogenates. These Ringer homogenates gave an activity towards glucose of 0.64 ± 0.07 mgm. glucose disappearing per millilitre homogenate in 2.5 min. (13 rats). The phosphorylation rates of the other sugars, measured under the same experimental conditions, relative to that of

Fig. 1.