THE REACTION OF H-SUBSTANCE WITH RABBIT ANTISERA

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(With 3 Figures in the Text)

Quantitative methods have been used in several investigations by Kabat and his colleagues (1945–50) in the study of serological relations of various preparations of blood-group substances. The investigations have taken two forms: (1) the amounts of antibody nitrogen precipitated from a standard volume of an antiserum by varying amounts of the blood-group substances have been plotted against the amount of blood-group substance added; (2) the hexosamine has been measured in the precipitates formed with antiserum in the zone of antibody excess and the ratio of hexosamine precipitated to hexosamine in the blood-group substance added has been calculated; the amount of methyl-pentose precipitated has also been measured (Kabat, Baer & Knaub, 1949) and the ratio of methyl-pentose precipitated to methyl-pentose added has been calculated.

From the curves obtained by method 1 it can be inferred: (*a*) that a substance x is identical with substance y, against which the antiserum was prepared, or (*b*) that x is closely related to y or is a mixture of y with some contaminant, or (*c*) that x is related less closely to y.

If the ratio found by method 2 is unity, it can be inferred that all the hexosamine in x is contained in substances that precipitate with antibodies in the anti-y serum. This result was specially significant when the antiserum was from a woman of group O who had been immunized to group A substance during pregnancy (Kabat, Baer, Day & Knaub, 1950).

The substance which was originally thought to be the O-group substance, product of the O-gene, is now considered to be a basic substance common to human red blood cells and it has been named H-substance (Morgan & Watkins, 1948). Morgan and his colleagues have isolated preparations of this substance and have immunized rabbits with it. We have studied the reaction of preparations of the H-substance with these sera.

MATERIALS AND METHODS

H-substances

These were prepared by Annison & Morgan (1952); the following details have been supplied by Prof. Morgan:

H 93/27. Hexosamine (as glucosamine base), 31 %; fucose, 14 %; N, 5·3 %; α-amino-acid N, 41 % of total N. This preparation is considered to be fairly homogeneous.

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H 74/54. Hexosamine, 24 %. This preparation contained some Le^a and almost certainly some Le^b substance, possibly as much as 25 %.

Antisera

Rabbits were immunized with preparations H 93/27 (sera 51, 56 and 303) and H 74/54 (serum M_3) after the materials had been combined with a conjugated protein recovered from the somatic antigen of *Shigella Shigae* (Morgan, 1943; Morgan & Watkins, 1944; Rainsford & Morgan, 1946).

Precipitation with antisera

Mixtures of antiserum, antigen and saline up to the desired volume were mixed and left at 38° C. until floccules had appeared in all the mixtures; they were kept at 4° C. for 3 days; the mixtures were then centrifuged. The precipitate was dispersed in ice-cold saline and centrifuged; this washing was repeated once.

Estimation of protein

The protein in the precipitates was estimated by the method of Heidelberger & MacPherson (1943); by this method we can measure 20μg. of protein (corresponding to about 3μg. of N). The method was calibrated with solutions of rabbit γ-globulin prepared by Kekwick's (1940) method slightly modified (Marrack, Hoch & Johns, 1951) in which the protein was estimated by a micro-Kjeldahl method; it was also checked against estimates (by Conway's (1947) method) of protein in the precipitates formed by protein antigens with antisera. We have found it preferable to add the Folin-Ciocalteau reagent in one portion (not dropwise), from a syringe, to the protein solution in the colorimeter cuvettes and to mix by inversion immediately after adding the reagent. The results with this technique are more consistent than those obtained when the reagent is added by drops, although the depth of colour formed is slightly less.

The depth of colour given by H 93/27 was negligible; the colour given by 1 mg. of H 74/54 was equivalent to that given by 0·08 mg. of γ-globulin. If the substance that gives this colour is precipitated, the calculated amounts of γ-globulin in the precipitates formed by H 74/54 with antisera are too high by amounts of the order of 2 %.

Corrections for the solubility of the precipitates were made by the method used by Bendich, Kabat & Bezer (1946).

Estimation of hexosamine

The method used was fundamentally that of Elson & Morgan (1933) with various modifications on the lines of those suggested by Kabat & Mayer (1948), Palmer, Smyth & Mayer (1937), Blix (1948) and Johnston, Ogston & Stanier (1951). All reagents were exhaustively purified with the exception of ethanol; it was found that commercial 'absolute' ethanol gave results in all respects identical with those given with carefully purified material. We found that the two samples of H-substance required widely differing hydrolysis times. With H 93/27 no significant increase in hexosamine content was obtained by prolonging the time of hydrolysis beyond 3 hr., whereas H 74/54 required at least 5½ hr. to give maximal values. Similarly, with mixtures of non-immune γ-globulin and H 93/27 a time of 4½ hr.

was ample; for the same protein and H 74/54 at least 6 hr. was required (see Table 1). The hydrolysis times employed were therefore: for γ-globulin alone, for

Table 1. *The effect of time of hydrolysis on amount of glucosamine found in various preparations*

Glucosamine is expressed as a percentage of the maximum value obtained.

	Glucosamine				
				Precipitate formed by antiserum with	
Hours hydrolysis	Rabbit γ-globulin	H 93/27	H 74/54	H 93/27	H 74/54
3	98	98	91	—	—
4·5	100	100	95	100	67
5·5	100	98	100	—	—
6	100	98	100	100	100
7	—	98	100	—	—
7·5	—	—	—	99·5	99·5

H 93/27 and for mixtures and precipitates involving H 93/27, 4½ hr.; for samples involving H 74/54, 7 hr. Glucosamine standards and reagent blanks were subjected to corresponding hydrolysis conditions; no significant destruction of glucosamine in the standards was observed.

The hydrolysate was evaporated to dryness *in vacuo* over sodium hydroxide and silica gel and the residue taken up in 1·0 ml. of water. 0·5 ml. of a 1·96 % solution of acetylacetone in 0·50 N-sodium carbonate was added, the tube resealed, and heated for 30 min. at 100° C. After cooling, the contents were transferred to a graduated tube and made up to 4·0 ml. with ethanol. 0·5 ml. of a 1·33 % solution of *p*-dimethylaminobenzaldehyde in ethanolic hydrochloric acid were added, and, after standing for 1 hr. at 25° C., the optical density determined at 525 mμ by means of a Hilger 'Uvispek' spectrophotometer.

We found the method of Dische & Borenfreund (1950) unsatisfactory. We were unable to get reproducible results, not only from day to day but even in replicate estimations run simultaneously. In recovery experiments in which glucosamine was added to γ-globulin the irreproducibility was such that we were unable to decide whether the method is or is not susceptible to interference from the globulin. Study of the experimental data given by Dische & Borenfreund reveals a number of disquieting features. For a series of glucosamine solutions of concentrations in the proportions 1·25, 2·5, 5, 10, 10, their values for $(D_{492} - D_{520}) \times 1000$ on non-deaminated materials are 6, 3, 24, 25, 0 (their Table 1, Exps. I and II). They state, moreover, that conditions of hydrolysis which yield almost theoretical values by the Elson & Morgan technique give low values with their indole method applied to mucopolysaccharides.

RESULTS

Estimation of hexosamine in precipitates

In the experiments in which the recovery of hexosamine in the antigen-antibody precipitates is estimated, the hexosamine is measured in the presence of a relatively large amount of γ-globulin. Kabat and colleagues (Bendich *et al.* 1946;

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Beiser & Kabat, 1951) estimated the apparent hexosamine content of γ-globulin and subtracted this from the total apparent hexosamine in the precipitate to get the hexosamine of the precipitate antigen. This calculation involves the assumption that the extinction coefficient of the coloured product formed by hexosamine, at the wave-length used in estimation, is not affected by the presence of relatively large amounts of protein.

We estimated the apparent hexosamine in four samples of rabbit γ-globulin (prepared as above) and one of human γ-globulin (Table 2). The amount in human γ-globulin (1·25 %) was slightly higher than found by Beiser & Kabat (1951) (1·12 % using 6·25 as the factor to convert N to protein). The amount in rabbit γ-globulin was considerably higher.

Table 2. *Apparent glucosamine in rabbit and human γ-globulin*

Sample	γ-globulin (μg.)	Apparent hexosamine (μg.)	Apparent hexosamine g. per 100 g. of protein
Rabbit A	4,050	80	1·97
	4,010	76	1·85
	4,050	79	1·95
	4,050	77	1·90
Rabbit B	26,000	510	1·96
Rabbit C*	500	9·63	1·93
	1,500	28·95	1·92
	1,250	24·1	1·93
	2,500	47·4	1·90
Rabbit D†	436	7·55	1·74
	872	14·8	1·70
	1,744	29·3	1·68
Human	800	10	1·25

* Each estimate is a mean of duplicates.

† Precipitate formed by bovine serum albumin with rabbit antiserum; contains 14 % bovine serum albumin.

The absorption curve of the colour formed in the Elson & Morgan reaction shows a maximum at a wave-length of 525 mμ (Fig. 1). The relationship between optical density at 525 mμ (band width 0·5 mμ) and concentration is linear for glucosamine, H-substance and γ-globulin up to optical densities of at least 2·5. We measured the optical densities obtained with glucosamine alone, H-substance alone, γ-globulin alone and with mixtures (Table 3) and found that the apparent hexosamine of mixtures of H-substance or of glucosamine with γ-globulin was more than the sum (apparent hexosamine of γ-globulin + glucosamine added as such or hexosamine of H-substance). In all experiments with mixtures of glucosamine with H-substance, the observed optical density did not differ from the calculated by more than 1 %. Seven experiments were made on mixtures of H-substances H 93/27 and H 74/54 with γ-globulin and twenty-eight on mixtures of glucosamine with γ-globulin. The ratio of glucosamine to γ-globulin in these experiments ranged from 0·5 to 5 parts of glucosamine to 100 parts of globulin. The mean difference between observed and calculated optical densities was +10·1 % with a range from 8·9 to 11 % (excluding one instance in which the

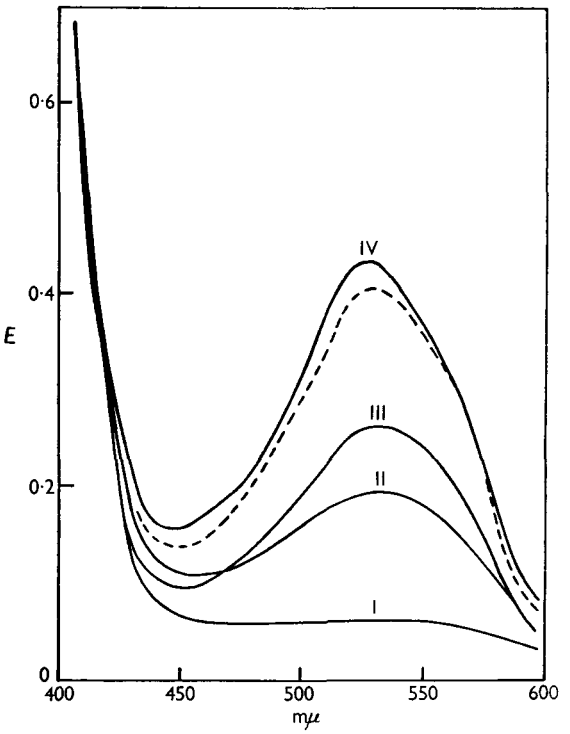

Fig. 1. Absorption curves of the colours produced in the Elson & Morgan reaction. Curve I, reagent blank. Curve II, 25 μg. of glucosamine. Curve III, 1250 μg. of rabbit γ-globulin. Curve IV, mixture of 25 μg. of glucosamine with 1250 μg. of rabbit γ-globulin. The dotted line is the calculated curve for mixture IV obtained by summation of curves II and III, with allowance for the blank.

Table 3. *Apparent hexosamine in human γ-globulin and mixtures of γ-globulin with H-substance and glucosamine*

	$E_{obs.}$	$E_{calc.}$
A. Glucosamine only:		
(1) 10 μg.	0·106	—
(2) 30 μg.	0·318	—
(3) 50 μg.	0·540	—
B. H-substance only:		
(4) 97 μg.	0·297	—
C. Human γ-globulin only:		
(5) 800 μg. approx.	0·101	—
D. Mixtures of 100 μg. H-substance with:		
(6) 10 μg. glucosamine	0·400	0·403 (1+4)
(7) 30 μg. glucosamine	0·615	0·615 (2+4)
(8) 50 μg. glucosamine	0·835	0·837 (3+4)
E. Mixtures of 800 μg. γ-globulin with:		
(9) 100 μg. H-substance	0·444	0·398 (4+5)
(10) 10 μg. glucosamine	0·237	0·207 (1+5)
(11) 30 μg. glucosamine	0·466	0·419 (2+5)
(12) 50 μg. glucosamine	0·732	0·641 (3+5)

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difference was 13·9 %). It can be seen from Fig. 1 that the optical density given by mixtures of glucosamine and γ-globulin exceeds the sum of the optical densities given by glucosamine alone and by γ-globulin alone at all wave-lengths below 570 mμ. The curves given by mixtures of H-substance and γ-globulin did not coincide at 570 mμ.

No variation of experimental conditions (e.g. concentration of reagents, pH control, time of heating) which we have tried has affected this difference significantly. Immers & Vasseur (1950) have stated that the interference due to glucose-lysine mixtures is avoided by using a 5–6 % solution of acetyl-acetone in a carbonate-bicarbonate buffer at a pH of 9·55. We have confirmed that this does reduce the glucose-lysine interference, but we find that it does not reduce interference due to γ-globulin. The carbonate-bicarbonate buffer, however, tends to give irreproducible results, since even in sealed tubes the pH drifts on heating.

We have made a detailed study of the effect of concentration and pH of the acetyl-acetone reagent, using concentrations from 1 to 10 % in plain carbonate and in carbonate-bicarbonate, borate-potassium hydroxide, and glycine-sodium hydroxide buffers at pH's ranging from 8 to 13. The pH for optimum colour development depends upon the type of buffer; thus, whereas for plain carbonate the pH optimum is at 9·55, for a borate buffer it is about 11. The depth of colour obtained with any of the buffers used was never as great as that given with plain carbonate, nor was there any reduction in the γ-globulin interference. We have, therefore, retained the use of a 1·96 % solution of acetyl-acetone in 0·50 N-carbonate. Higher concentrations of acetyl-acetone give slightly more sensitivity, but at the expense of a tendency to give turbidities with protein hydrolysates.

Einbinder (1950) treated the hydrolysed product with Darco G-60 before adding acetyl-acetone. We were not able to get a sample of Darco G-60, but tried a sample of acid-washed charcoal believing this to be similar to it. Any decrease in optical density as a result of this treatment was due to removal of glucosamine itself and not to removal of the interfering substance.

We have, therefore, made an additional correction in the calculation of recovered hexosamine (Table 4) to allow for this effect of the γ-globulin in the precipitates.

As an alternative method of measuring the amount of H-substance in the precipitates, we tried Dische & Shettles' (1948) method of estimating methylpentoses, using the CyR 10 technique. Two different samples of British DL-cysteine hydrochloride gave no colour with either fucose or rhamnose unless the concentration of the sugar was so high as to give a greenish colour with the sulphuric acid alone, when addition of cysteine produced a slight deepening of the colour. Both samples of cysteine nevertheless gave the pink colours described by Dische as given with pentoses and hexoses, and in the CyR 3 procedure gave the specified colour reactions with glucose, fructose and galactose. A sample of Eastman (U.S.A.) cysteine hydrochloride, however, with both fucose and rhamnose, did give the correct colour with a maximum absorption at about 400 mμ.

The colour given by the Eastman product was extremely faint; when the reaction was applied to the precipitates formed by H-substance 93/27 with antiserum the colour was completely masked by the brown hue caused by charring of the protein.

Table 4. *Recovery of hexosamine in antigen-antibody precipitates*

The different samples of serum M 3 differed slightly in degree of dilution after Seitz filtration.

Antiserum number and volume	H-substance added	Hexosamine in H-substance added (μg.)	Precipitate					Recovery (%)	Ratio, protein precipitated to hexosamine added	
			Protein (μg.)	Apparent hexosamine (μg.)	Apparent hexosamine corrected for					
					−10%	Protein	Solubility			
1·0	H 93/27	11·2	232	17·1	15·4	11·0	12·4	110	21	Antibody excess
1·0	H 93/27	22·5	272	23·5	21·2	16·0	17·6	78	12	Equivalence
1·0	H 93/27	22·5	252	24·7	22·2	17·4	19·4	86	11	Equivalence
M 3 (1) 1·0	H 74/54	46·5	905	72	65	48	51	110	19	Antibody excess
M 3 (1) 2·0	H 74/54	120	2020	145	131	93	98	82	17	Equivalence
M 3 (2) 2·0	H 74/54	68·4	1740	129	116	83	85	124	24·5	Antibody excess
M 3 (2) 2·0	H 74/54	114	2230	157	141	99	101	89	18	Equivalence
M 3 (2) 2·0	H 74/54	182	2500	194	175	127	130	71	—	Antigen excess
M 3 (3) 2·0	H 74/54	308	184	92	83	79·5	81·5	26·5	—	Antibody excess
M 3 (4) 2·0	H 74/54	44	990	72	65	46	47	107	22	Antigen excess

In the estimates of the recovery of antigen in the precipitates, using methylpentose as the marker (Kabat *et al.* 1949), the corrections ran as high as 30 %. With hexosamine as a marker the corrections for solubility and apparent hexosamine in the protein were considerable; the protein effect which we have found introduces yet another important correction. No conclusions should be drawn from differences of 10 % or less between the amount of hexosamine in the antigen added and the calculated antigen hexosamine in the precipitate.

Recovery of hexosamine of antigen added

The amount of hexosamine found in the precipitates formed with excess of antibody were in the neighbourhood of 100 % of the amounts in the H-substance added (Table 4). With the lower ratios of antigen to antibody, the recovery tended to exceed 100 %; considering that three large corrections are made, it is not necessary to envisage another source of error that we have not detected. We can infer that most of the hexosamine-containing molecules in preparations H 93/27 and H 74/54 give rise to antibodies in rabbits and are precipitated by the antibodies formed.

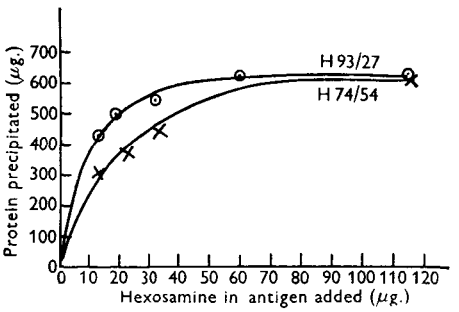

Fig. 2. Amounts of protein precipitated from 1 ml. of serum 303 by H 93/27 and H 74/54.

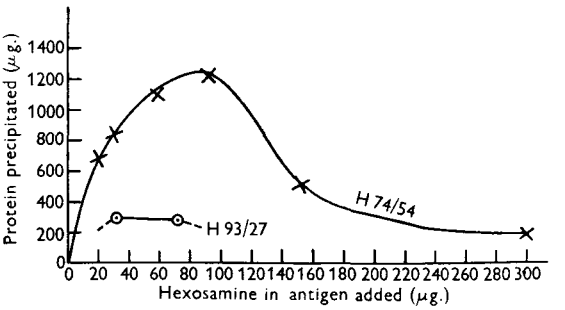

Fig. 3. Amounts of protein precipitated from 1 ml. of serum M 3 by H 93/27 and H 74/54.

Precipitation curves

Group substance H 74/54 precipitated practically all the antibody from the antiserum 303 prepared by immunizing with H 93/27 (Fig. 2); but more of H 74/54 than of H 93/27 was needed to precipitate a given amount of antibody. However, H 93/27 precipitated only about one-third of the antibody from serum M 3 prepared by immunizing with H 74/54 (Fig. 3). We may infer that H 74/54 contained an antigen similar to or identical with H 93/27 and some other antigen. H 74/54 contains less hexosamine than H 93/27; the ratio of protein to hexosamine in precipitates formed by H 93/27 at equivalence with homologous antiserum was 13, whereas the ratio in the precipitates formed by H 74/54 was about 19. It appears that the other antigenic substance in H 74/54 contains little or no hexosamine. The shape of the curve given by H 74/54 with antiserum M 3 (Fig. 3) resembled those found with protein antigens rather than those found with polysaccharide antigens; and it is noteworthy that the precipitates formed by H 74/54

with this antiserum were less soluble than the other precipitates studied. But Prof. Morgan assures us that H 74/54 contained no extraneous protein.

CONCLUSIONS

1. The depth of colour given in the Elson & Morgan reaction by mixtures of hexosamine or hexosamine-containing substances with γ-globulin exceeds that calculated from the depths of colour given by these substances independently.

2. The hexosamine in two samples of H-substance was recovered in precipitates formed with rabbit antisera in the zone of antibody excess.

3. All the hexosamine in these two preparations is contained in antigenic substances.

4. One of these preparations contains some antigenic substance that contains little or no hexosamine.

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REFERENCES

ANNISON, E. F. & MORGAN, W. T. J. (1952). *Biochem. J.* **52**, 247.
BEISER, S. & KABAT, E. A. (1951). *J. Amer. chem. Soc.* **73**, 3501.
BENDICH, A., KABAT, E. A. & BEZER, A. E. (1946). *J. exp. Med.* **83**, 485.
BLIX, G. (1948). *Acta chem. scand.* **2**, 467.
CONWAY, E. J. (1947). *Microdiffusion Analysis and Volumetric Analysis.* London: Crosby, Lockwood and Son.
DISCHE, Z. & BORENFREUND, E. (1950). *J. biol. Chem.* **184**, 517.
DISCHE, Z. & SHETTLES, L. B. (1948). *J. biol. Chem.* **175**, 595.
EINBINDER, J. (1950). *J. biol. Chem.* **185**, 725.
ELSON, L. A. & MORGAN, W. T. J. (1933). *Biochem. J.* **27**, 1824.
HEIDELBERGER, M. & MACPHERSON, C. F. C. (1943). *Science*, **97**, 405; **98**, 63.
IMMERS, J. & VASSEUR, E. (1950). *Nature, Lond.*, **165**, 898.
JOHNSTON, J. P., OGSTON, A. G. & STANIER, J. E. (1951). *Analyst*, **76**, 88.
KABAT, E. A., BAER, H., DAY, R. L. & KNAUB, V. (1950). *J. exp. Med.* **91**, 433.
KABAT, E. A., BAER, H. & KNAUB, V. (1949). *J. exp. Med.* **89**, 1.
KABAT, E. A., BENDICH, A. & BEZER, A. E. (1946). *J. exp. Med.* **83**, 477.
KABAT, E. A., BENDICH, A., BEZER, A. E. & BEISER, S. (1947). *J. exp. Med.* **85**, 685.
KABAT, E. A., BENDICH, A., BEZER, A. E. & KNAUB, V. (1948). *J. exp. Med.* **87**, 293.
KABAT, E. A. & BEZER, A. E. (1945). *J. exp. Med.* **82**, 207.
KABAT, E. A. & MAYER, M. (1948). *Experimental Immunochemistry*, p. 312. Springfield, U.S.A.: C. C. Thomas.
KEKWICK, R. A. (1940). *Biochem. J.* **34**, 1248.
MARRACK, J. R., HOCH, H. & JOHNS, R. G. S. (1951). *Brit. J. exp. Path.* **32**, 212.
MORGAN, W. T. J. (1943). *Brit. J. exp. Path.* **24**, 41.
MORGAN, W. T. J. & WATKINS, M. W. (1944). *Brit. J. exp. Path.* **25**, 221.
MORGAN, W. T. J. & WATKINS, M. W. (1948). *Brit. J. exp. Path.* **29**, 159.
PALMER, J. W., SMYTH, E. M. & MAYER, K. (1937). *J. biol. Chem.* **119**, 491.
RAINSFORD, S. J. & MORGAN, W. T. J. (1946). *Lancet*, **1**, 154.

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