QUANTITATIVE ASPECTS OF ANTIGEN-ANTIBODY REACTIONS

I. A THEORY AND ITS COROLLARIES[1]

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(With 11 Figures in the Text)

Many theories have been advanced with the aim of explaining and describing the quantitative course of antigen-antibody interactions, in particular the precipitin reaction.

In the early days of immunology Bordet (1939) and others regarded the immunological reactions as similar to colloidal phenomena such as mutual precipitation or adsorption, hence quantitative reasoning was difficult to apply. Even Ehrlich, however, used stoichiometrical concepts in his interpretations of the specific immunological reactions. Arrhenius & Madsen in 1907 pictured the toxin-antitoxin combination as corresponding to a reversible chemical reaction of the type $T+A \rightleftharpoons TA$ obeying the law of mass action. Many contemporary authors have advanced similar theories on the assumption that antigens (G) and antibodies (A) are multivalent with respect to each other and form compounds AG, A_2G, A_3G, etc. or AG_2, AG_3, etc., in general A_mG_n.[2] As regards the number of 'valencies' and the actual mechanism of the precipitate flocculation the views differ widely. Haurowitz (1939) and Hooker & Boyd (1942) assume the antibody to be monovalent and the antigen multivalent. Marrack (1938) and Heidelberger & Kendall (1935) assume both A and G to be multivalent, while Pauling (1940) regards A as bivalent and G multivalent. With respect to the flocculation there exist two theories different in principle: Haurowitz believes that compounds A_nG become insoluble when n is sufficiently high. On the other hand, Marrack, Heidelberger & Kendall and Pauling take the formation of insoluble floccules as an expression of a secondary combination of primary particles of the type A_nG to larger aggregates or complexes ('lattice'). In their simplest form these can be represented by $AG.A$, $AG.AG$, $AG.AG.AG$, etc., where the bonds can extend in all three dimensions. A more regular picture was recently sketched by Pauling.[3] The most extensive attempt so far to describe these reactions mathematically has been made by Heidelberger & Kendall in a series of papers starting in 1935. They apply the law of mass action to a series of bimolecular, consecutive reactions and obtain results which, on the whole, fit the experimental observations fairly well. From a theoretical standpoint, however, their fundamental assumptions are open to serious criticism (cf. Marrack, 1938, p. 169) and among practical defects can be mentioned that their formulae are valid only for the 'excess antibody zone' and have to be 'reversed' and modified in order to apply to the whole region of precipitation. Recently Heidelberger (1938) suggested definite empirical 'chemical formulae' for several antigen-antibody compounds which are based on analytical data for the composition of the precipitates and experimental values for the molecular weights of the components. Boyd & Hooker (1934, 1936) and later How (1939) also attempted to describe the 'equivalence point' precipitate in terms of molecular weights and stereometrical arrangement.

The aim of this paper is to suggest a new theory for the quantitative course of the antigen-antibody interactions, in particular valid for the precipitin reaction. Like its predecessors it uses the law of mass action, but, it is believed, does so in a simpler and more straightforward manner than the theory of Heidelberger & Kendall. The results also seem to have a more general significance.

The theory formulated is applicable not only to specific immunological reactions but also to precipitation reactions of unspecific nature as for instance the interactions between proteins and nucleic acid. In another paper, in collaboration with Björnesjö (1945), the author has described some experiments on precipitation reactions between thymonucleic acid and egg albumin or histone. The results obtained seem to be in good agreement with the theory proposed in this paper.

For a comparison between the theoretical predic-

[1] A preliminary communication was published in *Nature* (1943), **151**, 696.

[2] Burnet (1931) assumes adsorption superimposed on stoichiometric combination.

[3] Since the writing of this communication a new paper by Pauling *et al.* (*J. Amer. Chem. Soc.* **64**, 3003, 1942) has become available in this country in which a quantitative theory is given for a highly simplified system: $A+G \rightarrow AG+A_2G$. These authors also refer to quantitative papers by Hershey (*J. Immunol.* **42**, 455, 1941) and by Kendall (*Ann. N.Y. Acad. Sci.* **153**, 85, 1942) which, however, are not yet accessible in Sweden due to war conditions. In a recent paper by Hooker & Boyd (*J. Immunol.* **42**, 419, 1941) and by Boyd (*J. Exp. Med.* **75**, 407, 1942), 'they came to the conclusion that the "lattice" theory is probably not true' (cited from *Ann. Rep. Progr. Chem.* **39**, 222, 1942).

tions made below and actual experimental results for genuine 'specific' precipitation systems described in the literature, reference is made to the subsequent paper (Part II). On the whole, the agreement is quite satisfactory.

THEORETICAL CONSIDERATIONS

(1) *Principles employed.* The interaction between A and G is assumed to take place in steps with the formation of definite compounds. The system is supposed to be in reversible equilibrium according to the law of mass action. The quantitative treatment which will be given is in principle identical with the theory for the dissociation of a weak, polybasic acid. Precipitation will be considered due to the insolubility of one or more A-G compounds. The floccules formed are accordingly regarded as a mixture being dependent on the quantities of A and G which are brought together and on the 'dissociation constants' characterizing the system.

(2) *Assumptions.* (*a*) The antigen and the corresponding antibody are both regarded as single uniform substances. In accordance with Haurowitz and others it is assumed that the *antibody* (denoted as A) towards the antigen behaves as a *mono*valent substance and that the *antigen* (denoted as G) is *multi*valent, its maximum valency being N. Accordingly a series of compounds from AG, A_2G to A_NG can be formed besides the A and G left uncombined or 'free'.

The following simultaneous equilibria will now be established:

$A+G \rightleftharpoons AG$	with the dissociation constant	K_N
$A+AG \rightleftharpoons A_2G$	,, ,,	K_{N-1}
$A+A_2G \rightleftharpoons A_3G$	,, ,,	K_{N-2}
.....		
$A+A_{N-1}G \rightleftharpoons A_NG$	,, ,,	K_1

By application of the corresponding mass action equations it will be possible to calculate the quantity of any compound A_nG as well as that of uncombined A and G. These calculations will be performed in § 3 below.

(*b*) An important postulate is that the equilibria above are not influenced if one or several of the components loses its solubility and precipitates out. The consequences of such an assumption will be more closely considered in the discussion.

(*c*) With regard to *solubility* and *flocculation* it will be assumed that A and G in general tend mutually to precipitate each other as insoluble compounds with possible exceptions for certain compounds very rich in antigen or possibly also compounds rich in antibody, which will retain the complete solubility characteristic of the 'free' antigen and antibody.

In the applications of the theory given later in this paper only three possibilities will be considered:

Case X. All compounds AG, A_2G, etc., to A_NG insoluble.

Case Y. The compound AG soluble, the rest insoluble.

Case Z. The compound AG and A_NG soluble (i.e. the compounds richest in G and A respectively), the rest insoluble.

It should be emphasized that the precipitation is ascribed to insolubility of the 'primary' A-G compounds and not to a 'lattice' formation as thought by most previous authors.[1]

(3) *Application of the law of mass action* to the equilibria mentioned in the above section gives the following system of equations:

$$[A]\,[G]=k_N\,[AG], \tag{1·1}$$

$$[A]\,[AG]=k_{(N-1)}\,[A_2G], \tag{1·2}$$

$$[A]\,[A_2G]=k_{(N-2)}\,[A_3G], \tag{1·3}$$

$$\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$$

$$[A]\,[A_{(N-1)}G]=k_1\,[A_NG]. \tag{1·N}$$

After successive multiplication of the equations in the order (1·1) × (1·2) and (1·1) × (1·2) × (1·3), etc., and introduction of the abbreviations $p_1 \ldots p_N$, the system above can be rewritten

$$[AG] = \frac{[A]}{k_N}\,[G] = p_1[G], \tag{2·1}$$

$$[A_2G] = \frac{[A]^2}{k_N k_{(N-1)}}\,[G] = p_2[G], \tag{2·2}$$

$$[A_3G] = \frac{[A]^3}{k_N k_{(N-1)} k_{(N-2)}}\,[G] = p_3[G], \tag{2·3}$$

$$\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots$$

$$[A_NG]=\frac{[A]^N}{k_N k_{(N-1)} k_{(N-2)} \ldots k_1}\,[G]=p_N[G]. \tag{2·N}$$

Evidently equations (2) express the molar concentrations of the various A-G compounds formed as functions of their 'dissociation' constants and the concentration of 'free' or uncombined antibody and antigen. In order to allow explicit solutions of the $(N+2)$ unknown contained in equations (2) in terms of the total quantities of A and G initially mixed (denoted by $[a]$ and $[g]$ respectively) the system

[1] It is, however, conceivable that the insoluble particles remain in more or less stable colloidal state instead of forming larger, secondary aggregates which can flocculate to an actual precipitate. There is much experimental evidence of this; large excess of antigen seems especially to facilitate colloid formation, probably as a consequence of its protective action. For the sake of simplicity the influence of a marked solubility of the A-G compounds will not be discussed.

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has to be supplemented by the following two expressions:

$$[a] = [A] + [AG] + 2\,[A_2G] + \ldots + N\,[A_NG], \quad (3)$$

$$[g] = [G] + [AG] + [A_2G] + \ldots + [A_NG]. \quad (4)$$

Equations (3) and (4) in their turn may be transformed by substituting $[AG] \ldots [A_NG]$ by the corresponding right-hand members of equations (2) containing the abbreviations $p_1 \ldots p_N$. One then obtains

$$[G] = \frac{[a] - [A]}{p_1 + 2p_2 + 3p_3 + \ldots + Np_N}, \quad (5)$$

$$[g] = (1 + p_1 + p_2 + \ldots + p_N)\,[G]. \quad (6)$$

By the 'dissociation constants' and the known total quantities of antibody and antigen it is now possible from equations (2), (5) *and* (6) *to calculate the molar amounts of any A-G compound as well as the excess concentrations of uncombined A and G.* It is then easy to find the total amount of precipitate, etc.

A regular solution for the unknowns by substitution, etc. will become rather tedious, especially for high values of N. The actual numerical calculations are therefore best performed by a special method described below.

NUMERICAL CALCULATIONS

(1) *Calculation of the various A-G compounds.* In immunological practice there are two alternatives for mixing the antigen with the antibody: (1) the '*antigen dilution method*' (which Marrack and others also call the '*α procedure*') in which constant quantities of the antibody are mixed with varying quantities of the antigen, or (2) *the* '*antibody dilution method*' or the '*β procedure*' where the antigen is held constant and varying amounts of antibody are added.

The α procedure. (*a*) Some assumptions are made as to the numerical values of the equilibrium constants $k_1 \ldots k_N$ and of $[a]$, the constant total antibody concentration. (*b*) An arbitrary value of $[A] < [a]$ is postulated. (*c*) The values of the abbreviations p_1, p_2 to p_N are computed ($p_1 = [A]/k_N$, etc., cf. equations (2)). (*d*) $[G]$ is evaluated by aid of equation (5). (*e*) The values of the *A-G* compounds formed are now easily computed from equations (2) by multiplying $[G]$ by the appropriate p. (*f*) Finally the corresponding total antigen quantity $[g]$ is computed from equation (6). The processes (*d*)–(*f*) are then repeated, using each time a new $[A]$ value, preferably in a decreasing series, until sufficient data are obtained to allow a satisfactory numerical interpolation or the plotting of a smooth diagram over the desired range of reaction.

The β procedure. The calculations can be carried out as described for the α procedure with some slight modifications, namely that $[G]$ is computed from equation (6) where $[g]$ is known and that $[a]$, which is now a variable, is evaluated from equation (5).

All components of A and G have now been obtained in *molar* concentration units as a function of given antibody concentrations $[a]$ and antigen concentrations $[g]$. In general, however, one wishes to express the results on a *weight* basis instead of in molar units. The transformation from molar units to weight units can be easily performed by multiplying the molar quantities by the appropriate molecular weights. If these be α and γ for the antibody and antigen respectively, one obtains, for instance, $A = \alpha\,[A]$, $G = \gamma\,[G]$ and $AG = (\alpha + \gamma)\,[AG]$ or, in general, $A_nG = (n.\alpha + \gamma)\,[A_nG]$[1] (equations 7, 8).

(2) *Calculation of the total amount of precipitate and its content of A and G.* The total amount of the precipitate, denoted 'Tot. ppt.' is, of course, the sum of the various, single *A-G* compounds which in any individual case are assumed to be insoluble. As already mentioned on p. 228 only three simple alternatives will be considered in this paper.

I. '*The X case*', which is the simplest, where *all A-G* compounds are postulated to be practically insoluble. The total precipitate expressed in *molar* units will be

$$[\text{Tot. ppt.}] = [AG] + [A_2G] + \ldots + [A_NG], \quad (9{\cdot}1)$$

and for its content of A and G, denoted by [A ppt.] and [G ppt.], respectively, one obtains[2]

$$[A \text{ ppt.}] = [AG] + 2\,[A_2G] + \ldots + N\,[A_NG], \quad (10{\cdot}1)$$

$$[G \text{ ppt.}] = [AG] + [A_2G] + \ldots + [A_NG]. \quad (11{\cdot}1)$$

The corresponding *weight* units may be written

$$\text{Tot. ppt.} = (\alpha + \gamma)\,[AG] + (2\alpha + \gamma)\,[A_2G] + \ldots + (N\alpha + \gamma)\,[A_NG], \quad (9{\cdot}2)$$

$$A \text{ ppt.} \quad = \alpha\,([AG] + 2\,[A_2G] + \ldots + N\,[A_NG]), \quad (10{\cdot}2)$$

$$G \text{ ppt.} \quad = \gamma\,([AG] + [A_2G] + \ldots + [A_NG]). \quad (11{\cdot}2)$$

II. *The* '*Y case*'. Here the compound AG is assumed to be soluble, while all other *A-G* compounds are insoluble. Accordingly AG terms in equations (9), (10) and (11) should be excluded in this case.

III. *The* '*Z case*', where both AG and A_NG, i.e. the compounds richest in G and A, still remain soluble. Both AG and A_NG terms are, of course, now omitted in equations (9), (10) and (11).

[1] Symbols within brackets denote molar quantities, symbols without brackets denote weight units.

[2] Obviously one can also write more briefly: [Tot. ppt.] = [g] − [G] (equation 9·3); [A ppt.] = [a] − [A] (equation 10·3) and [G ppt.] = [g] − [G] (equation 11·3) and correspondingly on a weight basis: Tot. ppt. = α ([a] − [A]) + γ ([g] − [G]) (equation 9·4); A ppt. = α ([a] − [A]) (equation 10·4) and G ppt. = γ ([g] − [G]) (equation 11·4).

SOME THEORETICAL RESULTS

In order to demonstrate some features of the proposed theory a rather complete quantitative treatment has been devoted to a hypothetical system containing a tetravalent antibody ($N=4$) and having the 'dissociation' constants $k_1=10^{-3}$, $k_2=10^{-5}$, $k_3=10^{-7}$ and $k_4=10^{-9}$. The constant antibody or antigen concentration of the α and the β procedure respectively, has been fixed at 10^{-2} arbitrary weight units. The results are expressed in weight units with the simplifying condition that the 'molecular weights' of A and B are both equal to unity. In Figs. 1–9 the results are presented graphically.

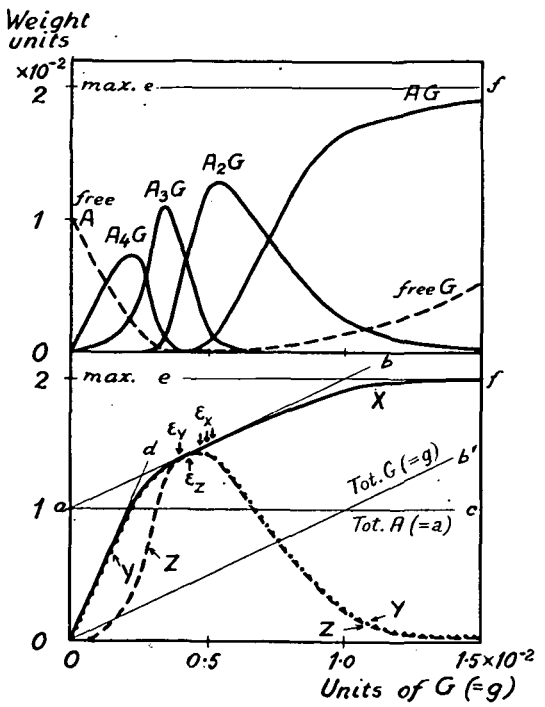

Fig. 1. Reaction between a constant amount of A with varying amounts of G (α procedure). *Upper part:* Amounts of single reaction products. *Lower part:* Total amounts of precipitate (X, Y and Z cases). Arrows referring to ϵ mark the equivalence points.

(1) *The A-G components* in the X, Y and Z cases are shown for the α procedure in Fig. 1 (β procedure, Fig. 5). An inspection of the figures shows that each can be divided into three regions, one where free A is present in a considerable excess, the 'excess antibody zone' and a corresponding 'excess antigen zone' (=the regions where the 'free A' and 'free G' curves are prominent respectively). The intermediate region, where only traces of free antibody and antigen exist uncombined, is 'the equivalence zone' or 'neutral zone'. The boundary between the zones is rather diffuse, the middle point of the equivalence zone, i.e. 'the equivalence point' may, however, be given a sharp definition which will be discussed later (p. 233).

In the excess A zone the 'higher' compounds A_4G and A_3G dominate and in the excess G zone A_2G and AG are mostly formed. This is, of course, to be expected from general mass action reasoning. The relative amount relations between the single compounds will, however, vary according to the total concentration a (or g)*[1] present. In an α procedure, for example, with a small antibody concentration, very little A_4G is formed which results in a diminished ratio A ppt./G ppt. as is shown by the ratio curves in Fig. 9—where a (or g)* is varied over a wide range from 10^{-2} to 10^{-7}. One may also express this as follows: the more dilute is the reacting mixture, the greater is the dissociation into the simpler AG compounds and the original constituents.

(2) *The amount of total precipitate* in the X, Y and Z cases is shown in the lower sections of Figs. 1 and 5 (α and β procedure respectively).

In both α *and* β *procedures* the X case curve (Figs. 1, 2, 5 and 6) has a somewhat hyperbolic form. It starts at g (or a when considering β procedure) equal to zero and rises rather sharply to bend in the ϵ-zone, to a more horizontal course and with further increase of g (or a)* it finally approaches a value which remains constant.

In the α *procedure*, the Y curve (Figs. 1, 3) coincides in its first part with the X curve. At the beginning of the excess G zone, however, a definite maximum appears and beyond this the curve again falls towards zero. This decrease is due to the fact that the compound AG is regarded as soluble and therefore not included among the insoluble products which constitute the total precipitate. The region of diminishing precipitate in the zone of excess G is regarded as corresponding to the 'antigen inhibition zone' or 'postzone' described in the immunological literature.

The Z curve of the α procedure (Figs. 1, 4) is similar to the Y curve but has in addition a 'prozone' or 'antibody inhibition zone' owing to solubility of the A-rich compound A_4G.

In the β *procedure* the Y curve (Figs. 5, 7) lacks a maximum and here the inhibition due to the excess of G appears at low a values which corresponds to the postzone of the α procedure. The Z curve (Figs. 5, 8) has an additional antibody inhibition zone and accordingly shows a marked maximum.

The position of the equivalence point will be discussed below.

(3) *The 'frames' of the Tot. ppt. curves.* In most of the diagrams a number of fine straight lines are plotted

[1] The parentheses with an asterisk refer to the β procedure, the main sentence to the α procedure.

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(cf. in particular Figs. 1 and 5). The significance of these will now be made clear. The horizontal line *a–c* represents the fixed weight of added *A* (added *G*)* while the ordinates below the line *O–b′* denote the quantities of variable *G* (or variable *A*)*. Its parallel, the line *a–b*, signifies the total *weight* sum of antigen and antibody added and is a 'frame' outside which the Tot. ppt. curve cannot extend. This line is of importance in the definition

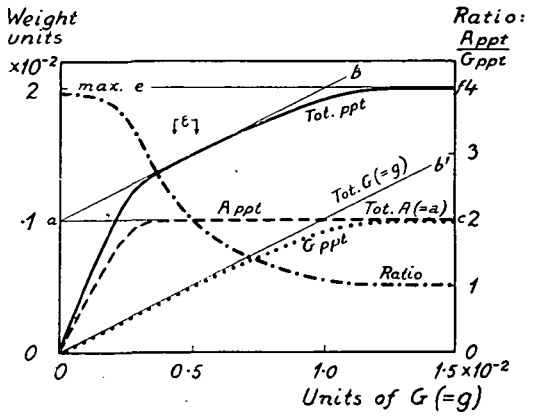

Fig. 2. Precipitin reaction between a constant amount of *A* with varying amounts of *G* in an αX case. ϵ marks the equivalence zone.

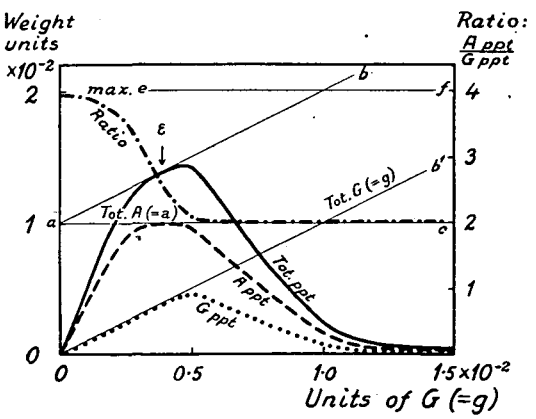

Fig. 3. Precipitin reaction between a constant amount of *A* with varying amounts of *G* in an αY case. ϵ marks the equivalence point.

of the 'equivalence point', as will be shown below. Two other 'frames' may also be constructed. First there is the maximum line *e–f*, which in the present case lies at the value 2 (or 5)* corresponding to the largest possible amount of AG (or A_4G)*. Secondly, there is the steep line *O–d* which forms the 'initial slope' to several of the discussed Tot. ppt. curves. In the units chosen this slope is equal to 5 (or 2)* again relating to the compound A_4G (or AG)*. Up to the intercept with the line *a–b* this slope coincides with the maximal amounts of A_4G (or

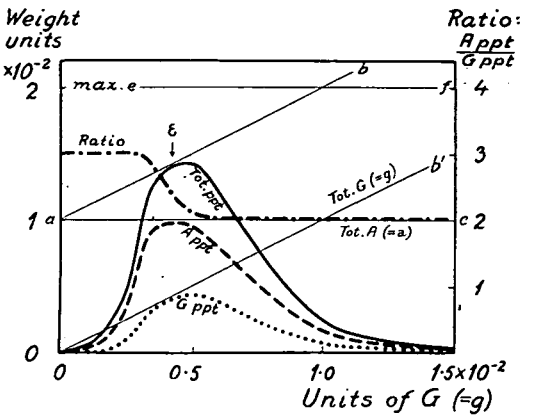

Fig. 4. Precipitin reaction between a constant amount of *A* with varying amount of *G* in an αZ case. ϵ marks the equivalence point.

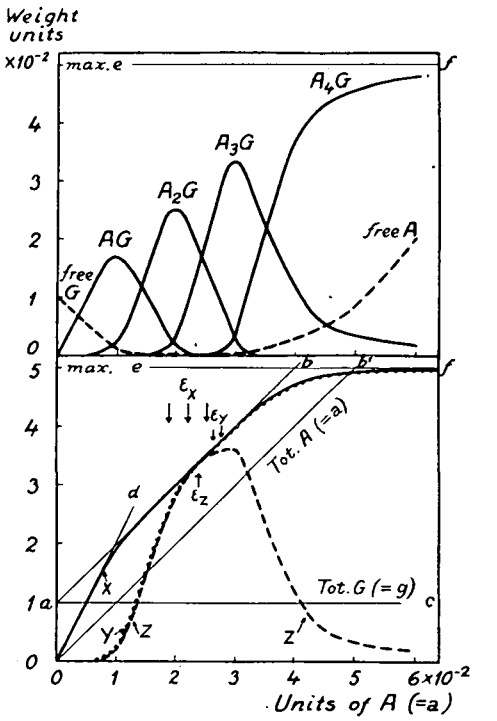

Fig. 5. Reaction between a constant amount of *G* with varying amounts of *A* (β procedure). *Upper part:* Amounts of single reaction products. *Lower part:* Total amounts of precipitate (*X*, *Y* and *Z* cases). Arrows referring to ϵ mark equivalence zones (points).

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AG)* which could theoretically be formed provided no other compounds were formed simultaneously.

(4) *The composition of the precipitate* with regard to the total content of antibody and antigen is demonstrated by the A ppt. and G ppt. curves of Figs. 2–4 and 6–8. As may be expected, both

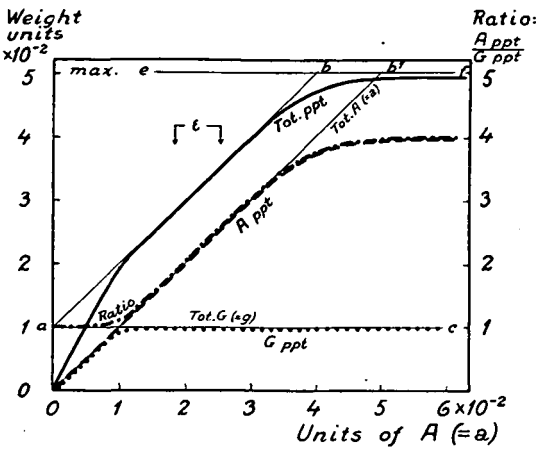

Fig. 6. Precipitin reaction between a constant amount of G with varying amounts of A in a βX case. ϵ marks the equivalence zone.

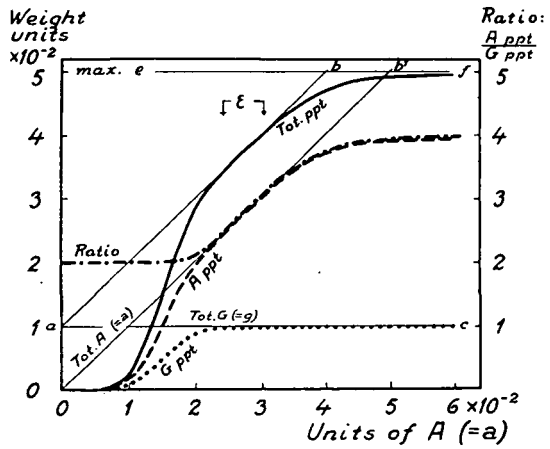

Fig. 7. Precipitin reaction between a constant amount of G with varying amounts of A in a βY case. ϵ marks the equivalence zone.

A ppt. and G ppt. increase with increasing g (or a)* until the equivalence zone is reached, beyond this, in the X case, the A ppt. (or G ppt.)* may remain approximately constant and equal to the total amount of A (or G)* added (the X case), or A ppt. and G ppt. may again diminish in the inhibition cases Y or Z.

The most convenient way of describing the composition of the precipitate is to use the *ratio A ppt./G ppt.*, which is also plotted in the figures referred to. The range of variation of the ratio depends on the case considered and the total concentrations of a (or g)*. It is widest in the X case, where it ranges

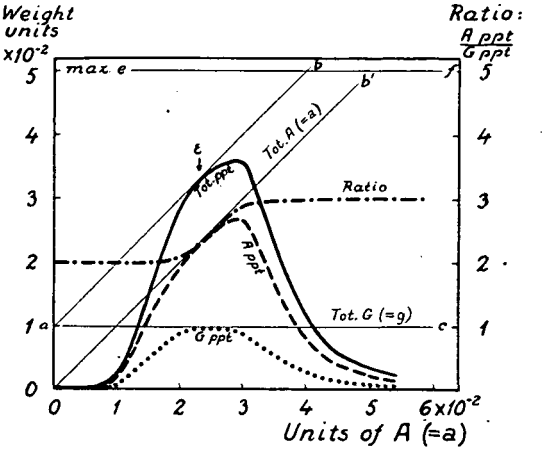

Fig. 8. Precipitin reaction between a constant amount of G with varying amounts of A in a βZ case. ϵ marks the equivalence point.

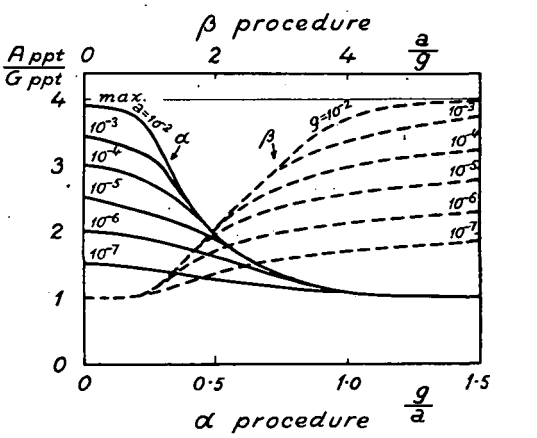

Fig. 9. The composition of the precipitates expressed as the ratio A ppt./G ppt. as a function of the total concentrations mixed. *Full lines* refer to α procedures. *Dashed lines* refer to β procedures. X case.

from nearly 4, where A is in excess, down to 1 with G in excess (cf. Figs. 2, 6). In the Y and Z cases the ranges are *c.* 4–2 and *c.* 3–2 respectively (cf. Figs. 3, 7 and 4, 8). The dependence of a (or g)* in the X case is demonstrated in Fig. 9. Similar diagrams apply to the Y and Z cases.

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The limiting values of A ppt./G ppt. were obtained from the expression

$$\frac{A \text{ ppt.}}{G \text{ ppt.}} = \frac{\alpha (p_1 + 2p_2 + \ldots + Np_N)}{\gamma (p_1 + p_2 + \ldots + p_N)}.$$

In the α procedure, at $g = 0$ we have $A = a$ and for $g = \infty$, $A = 0$. In the β procedure, at $a = 0$, $A = 0$ and $a = \infty$, $A = \infty$. When $A = 0$ all p terms but the lowest 'insoluble', p_1 or p_2, vanish and when $A = \infty$ all p terms but the highest 'insoluble' (p_N or p_{N-1}) vanish.

It is worth noticing that the molecular weights cancel in the expression (A ppt./G ppt. in excess A): (A ppt./G ppt. in excess G). For extreme excesses in a β procedure this expression has a limiting value determined only by the valency of the antigen, it is, for the X case $= N:1$, for the Y case $= N:2$ and of the Z case $= (N-1):2$. This relationship may be useful in experimental attempts to determine the valency of an antigen (cf. Björnesjö & Teorell, 1945).

(5) *On the equivalence or neutral point.* As already stated above, when a serial precipitation is carried out there appears an intermediate region where neither antigen nor antibody, or very small amounts thereof, can be detected in the supernatant solution. An attempt to define the 'midpoint' of this 'equivalence zone' will now be made. A closer consideration reveals that there may exist several alternatives for such a definition. A few of the least complex ones may be mentioned:

(1) The 'point' (= a certain a and g value) where (*moles of free* A) = (*moles of free* G). This definition is similar to that of the 'neutral point' of aqueous solutions in chemistry ([H] = [OH]) or to the isoelectric point definition of ampholytes.

(2) The point where (*moles of free* A): (*moles of free* G) = $(N\alpha + \gamma) : (\alpha + \gamma)$. Here tests on the supernatant with an excess of A or G would yield equal *weight* amounts of new precipitate as $A_N G$ and AG respectively.

(3) The point where the *weight sum* of unprecipitated material is at a minimum.

From a theoretical point of view (1) is the simplest definition. With reference to the procedures adopted in actual practice, however, the definition (2) may be preferable, when the difficulties in performing unambiguous supernatant tests are not considered. Furthermore, definitions (1) and (2) are difficult to reconcile with results obtained in experiments with systems of the Y or Z type, hence it seems justified to resort to alternative (3) which has some practical features in its favour and is equally applicable to the X, Y and Z cases.

In order to illustrate somewhat the significance of definition (3) some graphical considerations may be helpful:

In Fig. 1, for instance, which is plotted on a *weight* basis, the horizontal line a–c marks the total amount of antibody added, a, and the line O–b' the total amount of antigen, g. Evidently the line a–b represents the total sum of antibody + antigen. Now the region where the line a–b more or less touches the *Tot. ppt.* curve, here marked with ϵ's, constitutes the equivalence zone. Accordingly the following simple definition can be given, *the equivalence point* (ϵ-*point*) *is situated where the slope of the weight Tot. ppt. curve is equal to* 1 $\left(\frac{d\,(\text{Tot. ppt.})}{d\,(g)} = 1\right.$ and $\left.\frac{d\,(\text{Tot. ppt.})}{d\,(a)} = 1\right.$ for the α and β procedure respectively). A closer definition in terms of the equilibrium constants, etc. is of less interest because of its complexity.

From a practical point of view the proposed slope construction is of a certain value, because it may help to mark the approximate position and width of the equivalence zone in cases where only an analytically determined Tot. ppt. curve is available.

It should be expressively stated that the adopted definition, although convenient and useful in a case like the one treated here, may in other cases be of less value. Its principal weakness lies in its dependence on the molecular weights since it implies the use of weight units instead of molar units. It should be remarked, however, that the points computed according to definitions (1), (2) and (3) often approximately coincide.

An important corollary to these deductions is that *the* ϵ-*point can never correspond to the* '*maximal precipitation point*' because the slope at the latter is equal to zero, at the former equal to 1. For instance, in the X case of Fig. 2 the total precipitate at the ϵ-point is only about three-quarters of the maximum approached at great excess of antigen. In most cases with inhibition, however, the discrepancy between the two points is not so marked. This can be seen in Figs. 3, 4 and 8.

As regards the composition of the equivalence point precipitates it may be stated that no simple relation appears to exist. In general, the ratio A ppt./G ppt. is an average of the ratio values for extreme A and G excess (cf. the figures).

The completeness of the ϵ-*point precipitation* varies with the initial concentrations of antigen and antibody which are mixed. In the case here worked out the constant a (or g)* was taken as 10^{-2} which is of a higher order than the equilibria constants. At the ϵ-points of the cases given a practically complete precipitation occurs as may be judged from the close coincidence of a part of the line a–b with the Tot. ppt. curves. *If, however, lower* a *or* g *values are chosen* in an α or β procedure respectively, it may be found that *the* ϵ-*point precipitation becomes increasingly incomplete.* Fig. 10 illustrates this statement for the present case. The equivalence point ratio (A ppt./G ppt.) will simultaneously decrease. It can also be shown that the ratio a/g at the ϵ-point shifts when other values are chosen for the fixed antibody or antigen concentration.

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DISCUSSION

A theory has been presented which was based on the concept that the antigen-antibody interactions are governed by the same principles as the dissociation of a polybasic acid, say H_NT, where the antibody corresponds to the hydrogen ion H, and the antigen corresponds to the anion T. The quantitative aspects of the theory implied a straightforward application of the law of mass action. Before a comparison is made between the consequences of the theory and the available experimental results[1] it seems necessary to discuss some points in the theory which are of special importance.

(1) *The validity of the law of mass action and the irreversibility.* The essence of the present theory is the concept that the various compounds $A_NG \ldots AG$

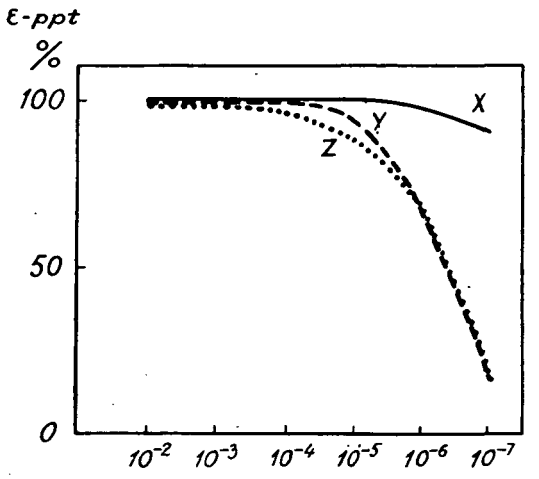

Fig. 10. Diagram valid for an α procedure and showing the amount of equivalence point precipitate [expressed in percentage of the highest possible amount (i.e. $a+g$)] as a function of the total A concentration (a).

and free A and G are present in a *reversible* equilibrium system obeying the law of mass action. This concept is in all probability applicable during the first moments after the mixture of A and G, before the precipitation has commenced. The application of the law of mass action to the precipitated system, however, seems to imply certain difficulties (even if the solubility product definition is employed), because, according to current views, the concentrations of undissolved particles should not enter into the mass action equation. Justified as this view is for inorganic precipitate systems when the particles are more or less dense or crystalline, it does not necessarily apply to the macromolecular systems involved in the antigen-antibody reactions. It may be conceivable that this type of systems exhibits a special form of structure of the aggregates in which the majority of the 'bonds' or 'receptor groups' are more or less 'buried' in the interior of the fabric formed by the giant molecules or micelles, this fabric, however, being so 'loose' that the bonds or groups will be freely accessible for interaction with molecules in the dissolved state. As an argument in favour of the proposed fabric structure may be taken the observation that the enzymic activity of beef catalase is not impaired by combination with its antibody (Campbell & Fourt, 1936). The reaction velocities in such a 'fabric' system will probably be highly reduced owing to a slowing up of the diffusion velocities within the fabric. The views developed would conform very well with the markedly bad reversibility (and solubility) often observed when extra antigen or antibody is added to a specific precipitate (cf. the so-called 'Danysz phenomenon' and related results of 'serial addition' which are often interpreted as due to 'irreversibility'). Many of the systems investigated are, however, at least slowly reversible.[1]

Thus the law of mass action would retain its formal validity also in the heterogeneous precipitation system although the numerical values of the constants may be different at different stages of the reaction. It would probably be more correct to speak of pseudo-equilibria and regard the equilibrium constants as a kind of 'average' or 'apparent' constants. At any rate it is likely that the equilibrium constants used above apply in the main to the initial stages of the reaction which probably determine the final composition of the system.

(2) *The effect of diluting the reacting mixture of antigen and antibody* has been found in practice to be small, which has been interpreted as a sign of small solubility or negligible dissociation of the compounds into their constituents (cf. Marrack, 1938, p. 146 for review). Heidelberger & Kendall (1935) have emphasized that the amount and composition of the precipitate depend only on the relative amounts of antigen and antibody present and not on their concentrations. Over a moderate range of dilutions (<tenfold) this may be in accordance with our theory, since the ratio A ppt./G ppt. remains rather constant, as can be inferred from the ratio diagram, Fig. 9. The amount will also remain rather constant, because the decrease in concentration is approximately compensated by the increase

[1] See the subsequent paper.

[1] Heidelberger & Pedersen (1937) reported that ultracentrifuge studies of a solution of an egg albumin system in the beginning of the excess antigen zone demonstrated the presence of several compounds with high sedimentation constants. With further excess of egg albumin there was a shift toward fewer compounds with smaller molecules and *several days* would have been required for the attainment of final equilibrium (personal communication by Dr K. O. Pedersen).

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in volume. At higher dilutions of the reactants, however, the precipitation will be incomplete, since the dissociation will be more dominant (cf. Fig. 10).

(3) *On the numerical values of the equilibria constants.* In the examples of the theory presented the 'first' constant k_1, belonging to the dissociation equation of the 'highest' compound $A_N G$, has been assigned a higher numerical value than the succeeding constants k_2, k_3, etc. This is in agreement with the conditions valid for polybasic acids. The numerical values chosen, $k_1 = 10^{-3}$, $k_2 = 10^{-5}$, $k_3 = 10^{-7}$ and $k_4 = 10^{-9}$, are quite arbitrary. A closer investigation shows, however, that the general shape and course of the curves and the conclusions need not be markedly changed if other values of the k's are selected. A wider 'spreading' of the k's leads to sharper bends in the curves and a closer approach to the 'frame' lines discussed in a previous section.

At present it seems impossible to estimate the order of the consecutive equilibrium constants in the natural antigen-antibody systems. In the simplest case one may perhaps regard the successive combinations of A with all the N reactive groups of G as independent processes. For such a case (polybasic acids), Wegscheider (1895) and later Adams (1916) have already deduced that the ratio between consecutive constants can be determined by statistical reasoning (see Britton, 1942, p. 241 and in particular J. Bjerrum, 1941, for these theories). Stenhagen[1] has derived the following simple formula for the ratio between any dissociation constants k_p and k_q of an N-basic acid behaving 'statistically'

$$\frac{k_p}{k_q} = \frac{q\,(N-p+1)}{p\,(N-q+1)}.$$

Applied to a tetravalent system one obtains

$$k_1 : k_2 : k_3 : k_4 = 1{\cdot}6 : 0{\cdot}60 : 0{\cdot}27 : 0{\cdot}10.$$

Such a 'statistical case' of an antigen-antibody reaction (Y case) was worked out in the preliminary communication of this theory (Teorell, 1943). It appears probable that systems with 'long chain' or 'thread-like' antigens (viruses) may approximate to statistical cases. Here the 'reactive centres' may have ample opportunity of operating independently of each other.[2]

(4) *On the valency of the antibody and antigen.* It should be pointed out that the quantitative treatment presented above does not necessitate any assumptions whatsoever with regard to the *nature* of the antibody-antigen 'valency bonds', a problem which will therefore not be considered here. Some discussion concerning the stereochemical conditions is required, however, in order to justify the assumptions made with regard to the *number* of valencies on the reactants (the term 'valencies' is taken in a wide sense to include ordinary chemical forces and 'weak' forces). The supporters of the 'lattice' theory picture the precipitate formed in the precipitin reaction as a network of antigen and antibody molecules (Heidelberger & Kendall, Marrack, Pauling). They must therefore postulate that both antigen and antibody are multivalent, a view that seems to be corroborated by experiments on 'built up' films[1] (cf. Porter & Pappenheimer, 1939). Our present knowledge of the protein structure of the antibodies also points towards polyvalence. Thus it is very probable that the antibody molecules *as such* are polyvalent. This fact does not necessarily imply, however, that these molecules *behave* towards antigen molecules as polyvalent. On the contrary, owing to steric hindrance, it is very likely that of the available valency bonds only very few can engage

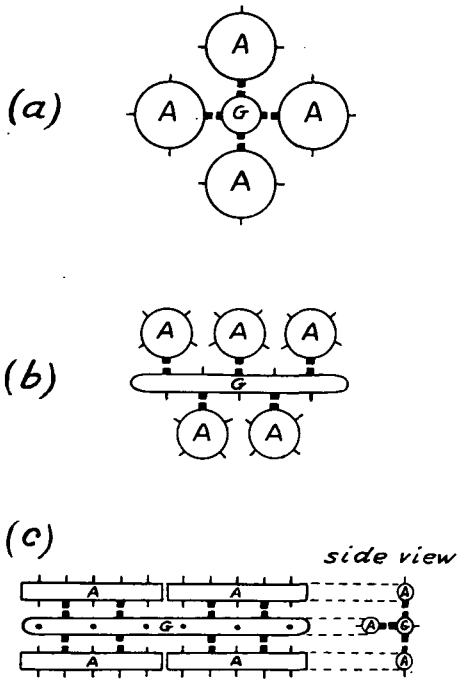

Fig. 11. Schematical pictures of some hypotheses on the structure of antigen-antibody compounds. (*a*) The antigen is a small, 4-valent 'molecule'. (*b*) The antigen is an elongated particle. (*c*) Both antigen and antibody have elongated forms. The 'effective' bonds are in heavy print.

with an antigen molecule. Indeed, it seems probable that, during the initial phase of an antibody-antigen reaction, only one bond per antibody molecule is 'effective' toward the antigen molecule as schematically shown in Fig. 11. Only the 'effective' valencies are drawn as thick lines. (The unoccupied bonds may perhaps participate in the secondary

[1] Personal communication.

[2] Kleczkowski (1941), working on plant viruses, found antigen 'N:s' from 12 to about 1000.

[1] This type of experiment involves adsorption processes at surfaces which may 'unfold' the native molecules. Hence the results must be interpreted with caution.

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flocculation or may be active in the so-called agglutinin reaction or in the complement fixation.)

In conclusion it seems justified to assume an 'effective' *mono*valence of the *antibody* as was done in the theoretical considerations above. This assumption is in agreement with the views advanced by other investigators (Haurowitz, Hooker & Boyd) and is substantiated by the fact that the theoretical results derived in this paper seem to agree fairly well with the experimental evidence available as will be shown in the subsequent communication.

Monovalence of the antibody does not, however, constitute a *conditio sine qua non* for the present theory. An attempt to analyse the problem seems to show that it may also be possible to develop a theory for the case when the antibody is polyvalent. The mathematical obstacles, however, seem to be great and therefore no effort will be made here to perform such an analysis.

All the evidence indicates that the valency of the *antigen*, N, is higher than one and in the examples it was taken to be four. An increase in N does not change the type of the reaction curves here pictured, although the ratio curve (A ppt./G ppt.) acquires a more extended course.

(5) *The molecular weights* of both A and G were here given a value of unity in order to facilitate the computations. The general consequences will not be markedly altered by assigning different molecular or equivalent weights to A and G, although the bends in the weight curves will appear sharper or more rounded as the case may be. The lower the molecular weight ratio, α/γ, the closer is the approach of the Tot. ppt. curve to the G ppt. curve. At the same time the ϵ-point, as defined above, will shift towards the origin.

SUMMARY

1. A quantitative theory has been developed for the reactions between antigens and antibodies, applicable in particular to the precipitin reaction. The theory is equally applicable to unspecific precipitations, for instance the reaction between proteins and protein precipitating agents such as nucleic acids, etc.

2. The theory is based on the concept that the antigen-antibody interaction is governed by the same principles as the dissociation of a polybasic acid, H_NT, where the antibody corresponds to the hydrogen ion H, and the antigen to the anion T. In their mutual reactions the antigen G is supposed to be polyvalent and the antibody A monovalent: hence the result of the interaction is a *mixture* of compounds A_NG, $A_{N-1}G$, ..., AG.

3. A mathematical deduction is performed starting from the law of mass action, which leads to expressions for the amounts of total precipitate and its constituents in terms of the quantities of antigen and antibody which were mixed and the known equilibria constants.

4. A numerical example is given for a tetravalent antigen. Three cases are represented in graphical forms: the 'X case' without inhibition zones, the 'Y case' with one and the 'Z case' with two inhibition zones. Certain characteristics are described and the significance of the 'equivalence point' is analysed.

5. A discussion deals with the irreversibility in relation to the Danysz phenomenon and the dilution effect, and finally the parameters of the theory are more closely considered.

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