Synthetic Immunochemistry.

A LECTURE DELIVERED BEFORE THE CHEMICAL SOCIETY ON NOVEMBER 16TH, 1939.

By C. R. HARINGTON.

WHEN the body is invaded by a poisonous substance of simple chemical nature it frequently responds by calling into play one or other of certain well-recognised mechanisms of detoxication. Thus it is a familiar fact that in the course of ordinary metabolism toxic phenolic substances which are formed by bacterial action in the intestines and are thence absorbed into the blood stream are detoxicated by conjugation in the liver with sulphuric acid or glycuronic acid into esters which are non-toxic and can be easily excreted by the kidney; again, poisonous aromatic acids such as benzoic acid are commonly detoxicated by conjugation with the amino-acid glycine to form the harmless excretory product, hippuric acid.

It is my purpose in this lecture to present the evidence that the reactions of immunity represent defence mechanisms on the part of the body which do not differ in principle from the simple chemical detoxications which I have mentioned, although they show in detail an enormously greater subtlety and complexity in proportion to the greater complexity of the agents which bring them into action; I hope further to show that there is a possibility of utilising the known facts of immunochemistry to develop a new means of attack on certain fundamental problems of pharmacology.

The person who has not made a special study of the science of immunity will probably think of immune phenomena in terms of his everyday experience; he will know, for instance, that if he has had an attack of an infectious disease such as measles in childhood, he is, practically speaking, safe against a subsequent attack of the same disease; he will know also that in some cases such security against infection can be artificially produced, as in vaccination against small-pox, inoculation against typhoid fever and immunisation against diphtheria; he may finally be aware that protection, which is of a transient character only, can be afforded by introducing into the body the blood serum of an animal or human being who has been infected, a phenomenon which is well illustrated by the modern prophylactic treatment of measles with the blood serum of patients recently convalescent from the disease.

If he has thought on these lines he will have considered some of the most fundamental features of the process of immunisation. The immunity which is produced by an attack of a disease or which can be produced artificially by deliberate introduction into the body of dead or attenuated pathogenic micro-organisms or of their metabolic products, as in the various methods of inoculation, is known as active immunity; its production shows that invasion of the body by such micro-organisms or their products can call into play a defence mechanism by means of which the pathological effects of the invading organism (or product) can be overcome. The fact that transient protection can be conferred on an individual by the blood serum of another who has defeated the infection (passive immunisation) further indicates that the defence mechanism is transferable and therefore presumably involves an actual change in the blood of the infected person.

That a real change does take place in the blood of a person infected with a pathogenic micro-organism can in fact usually be demonstrated by one or other of a series of test-tube reactions. A familiar example is the agglutination reaction; thus typhoid bacilli will, under proper conditions of p_H and electrolyte concentration, form a stable suspension; if such a suspension be mixed with normal blood serum its stability will be undisturbed; if however it be mixed with the serum of a patient who has, or has recently had, typhoid fever, or who has been inoculated against typhoid the suspension will become unstable and the organisms will agglutinate and eventually precipitate. In other cases the immune serum may be found to form a precipitate with a soluble fraction or metabolic product of the infecting organism; in still others the infecting organisms may be dissolved by immune sera prepared against them. The last-mentioned reaction is more complex than the other two in that it involves the participation of a heat-labile substance which is

present in normal serum and is known as complement; this is possibly of enzymic nature and it possesses the property of being "fixed" or removed from the sphere of action whenever reaction occurs between an immune serum and the substance which gave rise to its production, a property which makes it of the greatest value for the detection of immune reactions which cannot be demonstrated in the other more obvious ways. Since the details of the complex reaction of complement fixation have no direct bearing on the main theme of this lecture, they will not be further discussed in this place.

Now so far we have only considered immune reactions which are brought about by the introduction into the body of pathogenic micro-organisms or their metabolic products; many other substances, however, are able to elicit reactions of a precisely similar type and the general term antigen is used to describe any substance which, when introduced into the deeper tissues of the body, is able to call into play a reaction which can be demonstrated by an altered behaviour of the blood and tissue fluids to this substance; since it will be clear from what I have already said that the process involves a chemical alteration in the blood or tissue fluid, the altered behaviour may be hypothetically identified with a new or altered constituent of these fluids which may be designated an antibody.

In the most general manner it can be stated that all material of a protein character which is foreign to the body is potentially antigenic and conversely that it is still uncertain whether an immune reaction has ever been produced against a substance which is free from protein. Thus the injection of foreign cells, such as the erythrocytes of another species, gives rise to an antiserum which has the power to dissolve these cells; on the other hand the antiserum obtained by injecting a soluble foreign protein such as egg-albumin shows specific precipitation with the antigen up to high dilutions.

It is convenient at this stage to summarise the common ways in which the antigen–antibody reaction may be demonstrated in the test-tube (see Table I).

TABLE I.

Reactions observed *in vitro* between antigens and antisera.

Stage of antigen.	Antigen–antibody reaction observed.	Example of antigen.
Dissolved	Precipitation	Foreign protein
Dissolved	Neutralisation	Bacterial toxin
Particulate	Agglutination	Pathogenic bacteria
Particulate	Lysis	Foreign erythrocytes

If the antigen is dissolved, as in the case of a soluble foreign protein, the reaction will frequently be demonstrable as a precipitation; if in addition the antigen has poisonous properties (*e.g.*, diphtheria toxin), these will be neutralised by admixture with an appropriate amount of the antiserum. If the antigen is particulate, one of two things may occur, examples of both of which have already been mentioned; thus an antigen consisting of a suspension of pathogenic bacteria may be agglutinated, whilst one consisting of a suspension of foreign erythrocytes may be dissolved.

The question next arises as to the nature of the participant in these reactions which is formed in the body, that is to say, the antibody; what sort of a compound is it, where does it come from, and is it one and the same type of compound in all cases or do different types of antibody exist, corresponding to the different immune reactions? The answers to these questions are fraught with some uncertainty, but it can at least be stated with a fair degree of confidence that antibodies are proteins and that they are not only constantly associated with the blood serum globulin but that they are probably altered forms of that protein. The question of the unity or diversity of types of antibody is still more uncertain; in this connection it is only possible to say that the antigen–antibody reaction presents the ordinary features of a colloid–chemical interaction, and that it is at least possible that all antibodies are of the same type, the different manifestations of their action being no more than a reflection of the difference in physical state of the antigens with which they react.

There is, however, one feature of immuno-chemical reactions which sets them in a class apart from all others and that is their very high degree of specificity. In so far as pathogenic organisms are concerned this specificity is a matter of common knowledge;

it extends, however, through the whole class of antigenic substances, and it is with the factors which determine specificity that this lecture is chiefly concerned.

Before proceeding to the detailed discussion of this question it is necessary to amplify somewhat the general statement that all foreign proteins are antigenic; by the ordinary definition of the term protein this statement is not strictly true. It is clear that one necessary characteristic of an antigenic substance is that it should possess a very high molecular weight; nevertheless molecular weight as such does not seem to be the determining factor in the antigenicity of proteins; a protein of low molecular weight, such as egg-albumin, for instance, is a stronger antigen than the high-molecular casein. Rather does it seem that molecular integrity is the decisive requirement; thus it is difficult to imagine that in the early stages of the degradation by enzymic or other means of high-molecular proteins fragments will not be present which exceed egg-albumin in molecular magnitude; nevertheless such early stages of breakdown are invariably associated with the total loss of antigenic power.

The amino-acid composition of a protein may also be of importance in relation to its antigenic capacity. The great majority of natural proteins contain in their molecules most of the known amino-acids, although in varying amount; certain proteins, however, are almost or entirely deficient in one or more amino-acids and it therefore becomes possible to decide whether these amino-acids are essential components of an antigen. The one fact which emerges with certainty from studies of this kind is that the presence of the amino-acid tyrosine is essential.

Antigenicity among proteins is also influenced in a general way by the biological function which the proteins fulfil. In this respect the sharpest contrast is to be observed between what may be called the metabolic proteins on the one hand and the hormonal proteins on the other, the storage and structural proteins occupying an intermediate position. The metabolic proteins, as typified by the proteins of blood serum, exhibit both antigenic power and specificity in the highest degree; not only is the blood serum of one species strongly antigenic to other species to within very close degrees of relationship, but the individual proteins of the blood can also be easily distinguished from one another immunologically. Some overlap does of course occur, as, for instance, between man and the higher anthropoid apes, between the dog and the wolf, and between the horse and the ass; in such closely related pairs mutual antigenicity between species is weak or non-existent; in general, however, the specificity exhibited is astonishingly high. Storage proteins such as the casein of milk and structural proteins such as the protein of the eye-lens and keratins on the other hand show a different behaviour; they are indeed antigenic, even to the extent of acting as antigens in the species from which they are derived; on the other hand they exhibit little or no species specificity, antisera prepared, for instance, against eye-lens proteins from a number of unrelated species of animals being indistinguishable from one another; this lack of species specificity is obviously correlated with the fact that proteins of this type have biological functions which are common to the different species from which they are obtained. The extreme position is occupied by hormonal proteins, which not only fulfil the same function in different species but are themselves present in the deeper tissues of the body; the obvious example of this class is insulin, which, although it satisfies the criteria for an antigenic protein in so far as its molecular weight and amino-acid composition are concerned, exhibits no antigenic properties whatever. The evidence as to the antigenic properties of thyroglobulin is open to question owing to the difficulty of purification; it is at any rate not a strong antigen and it is possible that if it could be properly purified by crystallisation it would be found, like insulin, to be non-antigenic.

We may summarise, then, by saying that the present state of knowledge indicates that a foreign protein will be antigenic so long as it is intact in structure and so long as its complement of amino-acids includes tyrosine, provided always that it has no specialised hormonal function. Antigenic power and antigenic specificity will be quantitatively influenced by the normal function of the protein, being most highly developed in proteins which are involved in active metabolism and much less so in those concerned with storage and structure.

It is difficult to imagine any explanation of the sharp difference to be observed between the immunological specificities of proteins other than one based on variations in their chemical structure. It is highly probable that the structure of the protein as a whole plays a part in this matter; thus it is possible to distinguish immunologically between such closely related proteins as the albumins of the eggs of the duck and the hen; in this case no difference between the proteins is detectable by the ordinary methods of chemical analysis in so far as their amino-acid contents are concerned, and it is only by racemisation experiments that a suggestion is obtained that the relative arrangements of certain amino-acids are different in the two cases, so that it must be assumed that a small intramolecular rearrangement is sufficient to modify specificity. Much more definite information can be obtained, however, by studying the effects of various reagents on proteins in respect of the immunological reactions of the latter.

The molecules of all proteins contain certain functional groups which can be modified by the action of chemical reagents without disruption of the molecule as a whole; such modifications may alter the chemical and biological behaviour of the protein and may thus give an indication of the groups which are responsible for the specific properties of the latter in its original state. Thus it has been possible to deduce that the biological activity of insulin is determined in part at least by the phenolic groups of the tyrosine which it contains, since the action of reagents which modify these groups renders the protein inactive, and further examples of the same kind are known.

In order that experiments of this type should lead to valid deductions certain points have to be borne in mind. In particular it is essential that the action of the reagent employed should be specific; neglect of this requirement has led to much confusion. It is impossible, for instance, to draw any useful conclusions from the fact that the behaviour of a protein is altered by the effect of such reagents as acid and alkali which may attack many parts of the molecule; on the other hand examples will be given later in which clear evidence is available that the modifying reagent employed is confined in its action to certain specific groups; in such cases it is justifiable to conclude that any alterations in the behaviour of the modified protein are due to the modification induced in these groups. It is clear that any deductions made from the results of artificial modification of a protein will gain greatly in value if the modification is reversible in character; in at least one case such reversibility has been observed, namely, in the inactivation of insulin by iodination, followed by restoration of activity by deiodination of the inactive product; however, the immunological behaviour of proteins is affected by such extremely small chemical alterations that the ideal of reversible changes in immunological properties has not hitherto been attainable.

We may now consider certain specific reagents which have been profitably employed for studies of the sort under discussion. The first of these is formaldehyde. This reagent is commonly supposed to react with the amino-groups of proteins and so far as is known no other groups are involved; if a protein is treated with formaldehyde, its immunological specificity is found to be altered; the alteration, however, in this case is limited in extent, since the original species specificity is not abolished. If, for instance, horse serum globulin is treated with formaldehyde and the product is injected into a rabbit, an antiserum will be produced which will react strongly with formaldehyde-treated horse-serum globulin, weakly or not at all with untreated horse-serum globulin and not at all with formaldehyde-treated proteins of other origin.

Now if we employ a reagent which attacks protein more vigorously, we obtain a different result. A good example is afforded by acetic anhydride, which can attack not only amino-groups but the phenolic hydroxyl groups of the tyrosine and possibly also imino-groups in proteins. If a protein is completely acetylated by treatment with acetic anhydride, its immunological behaviour is altered in a much more fundamental way than when it is treated with formaldehyde. Acetylated horse-serum globulin, for instance, when injected into a rabbit, will give rise to an antiserum which will react not only with the acetylated protein used as antigen but also with a large variety of other acetylated proteins; that is to say, the change in immunological specificity induced by acetylation is so profound as to transcend species differences altogether. It is of some interest to

note the analogy between the profound effect of esterifying the phenolic groups of tyrosine on the immunological behaviour of proteins, and the similarly profound effect of such esterification on the biological activities of insulin and pepsin.

The methods of chemical modification of the protein molecule which, until quite recently, have been most fruitful in immuno-chemical studies have indeed been those involving a different sort of alteration to the tyrosine groups. The first reaction of this type to be considered is substitution with iodine. There is good evidence from other sources that treatment of a protein with iodine under proper conditions, *i.e.*, in concentrated ammoniacal solution in the cold, results in no significant alteration to the protein molecule other than substitution of the tyrosine groups with iodine in the 3 : 5-positions. Such iodination of a protein has an effect on its immunological behaviour which is of the same profound character as that of complete acetylation; an antiserum prepared against an iodinated protein will thus react not only with the antigen used in its preparation but with many other iodinated proteins. The same remarks apply to the alterations in the immunological behaviour of proteins which can be induced by other methods of treatment such as chlorination, bromination, and nitration, which, although less well-defined in their actions, probably attack in the main the tyrosine groups; in all cases a new specificity is introduced which is characteristic of the new group formed and transcends the limits of the species specificity of the original protein.

Most important of all, however, is the fact that proteins, by virtue of their content of tyrosine phenolic groups (and also perhaps to a less extent of their iminazole groups) are capable of coupling in alkaline solution with diazonium salts to give products which exhibit a new immunological specificity characteristic of the diazo-compound employed. By the use of this method, first introduced by Landsteiner, it has been possible to attach an enormous variety of compounds to proteins and to make a close study of the effects of variations in the groups attached on immunological specificity.

A few examples illustrating the application of this method may profitably be given. Thus tartranilic acid can be nitrated, and the nitro-compound reduced; the resulting amino-derivative may be diazotised and coupled with a protein; if this series of operations is carried out starting in turn from *d*-, *l*-, and *meso*-tartaric acids, we shall obtain three distinct proteins differing only in the stereochemical configuration of the groups which have been attached to them; comparison of antisera prepared against these three proteins shows distinct differences between them; precipitation reactions are indeed obtained between any one antiserum and all three antigens, but it can be shown by quantitative titration that the reaction between an antiserum and the homologous antigen (*i.e.*, the antigen against which it was actually prepared) is always quantitatively the strongest.

Experiments of this type can be carried out in the carbohydrate series. For instance, a series of *p*-aminophenylglycosides of different sugars may be prepared and, after diazotisation, coupled separately with a protein to give a series of derivatives again differing only in the stereochemical configuration of the sugar groups introduced; if these derivatives are used as antigens, it will again be found that their respective antisera can be quantitatively differentiated from one another, the ease of differentiation increasing with the magnitude of the stereochemical difference between the individual antigens.

I have given only two examples out of a very large number which are available; these will, however, suffice to illustrate the points of main importance, namely, that the new groups introduced have an overwhelming effect in determining the immunological behaviour of the derivative containing them and that the smallest variations in the nature of the groups are detectable by the study of immunological specificity.

Reality is given to the conception of the artificially attached groups actually determining the specificity of the protein derivatives by consideration of a reaction which is of great value in the study of immunological properties, namely, the serological inhibition reaction; this consists in the power of the compound constituting the determinant group to inhibit the specific precipitation between antigen and antiserum. If, for instance, the antiserum against a β-glucosidophenylazoprotein is incubated with excess of *p*-aminophenyl-β-glucoside before admixture with the antigen, the precipitation will not occur; the reaction is specific, in that similar inhibition cannot be obtained even with closely

allied compounds such as *p*-aminophenyl-β-galactoside, and is explained by the assumption that the excess of the determinant compound occupies the appropriate combining sites on the molecule of the specific antibody and thus prevents the latter from combining with the antigen itself to form an insoluble precipitate; on this assumption, therefore, the determinant groups in the artificial protein derivatives which we have been discussing are regarded as representing the actual participants in the linkage which is formed between antigen and antibody and hence are named hapten groups.

Now the rôle of these hapten groups in artificial derivatives of protein has its analogy in Nature. This is best illustrated among the bacterial antigens, and modern ideas on the subject have developed from the work of Avery and Heidelberger on the pneumococcus. Patients suffering from pneumonia excrete in the urine a substance which gives, up to very high dilutions, specific precipitation with antisera against the type of pneumococcus to which the infection is due; the same substance can be isolated from cultures of the organism and it has been proved to be of carbohydrate nature and in all probability to be identical with the capsular gum of the pneumococcus itself. The reaction is absolutely type-specific; that is to say, each type of pneumococcus produces a different carbohydrate, which will react only with antisera against its own type. These carbohydrates are not themselves antigens, since they will not, when injected, elicit the formation of an antiserum; it is clear, however, that they are decisive in determining the specificity of the bacterial antigen as a whole, and that they perform in the latter a haptan function strictly analogous with that which I have described for the simple hapten groups which may be artificially attached to proteins.

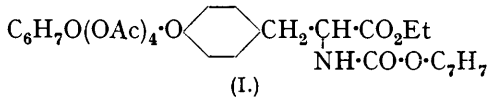
These observations are now known to apply not only to the pneumococcus but to the majority of other bacterial antigens, which seem indeed in general to be protein–carbohydrate complexes in which the carbohydrate hapten plays a determinant part in respect to specificity. The natural carbohydrate haptens so far known are for the most part substances resembling gums in constitution; that is to say, they are polymerised aldobionic acids. Remarkable experiments have been made with them which reveal the reality of the part which they play in immunological processes; for instance, type II pneumococcus polysaccharide has been converted into the *p*-aminobenzyl ether and the resulting derivative has been diazotised and coupled with horse-serum globulin; the complex so obtained, which contains the specific bacterial polysaccharide artificially attached to a protein which is entirely unrelated to the pneumococcus itself, has been used for the preparation of an antiserum, and the latter has been found to be capable of protecting mice against infection with the same type of pneumococcus. In one case recently it has been claimed that an effective antigen for the preparation of such a protective serum is a derivative formed by coupling a protein not with the complete bacterial polysaccharide but with the aldobionic acid forming the structural unit of this polysaccharide, so that a prospect begins to appear of the possibility of the synthesis of antigens which may be of value in the production of therapeutic antisera.

I now come to the description of some attempts, in which we have ourselves been interested, to contribute to knowledge in this field. The experiments which I have to describe fall into two groups, the first being concerned with the theoretical question of the essential nature of certain groups in proteins for the development of antigenic power, and the second with the attempt to apply the knowledge gained by the investigations of earlier workers which I have described to a type of experimental pharmacology.

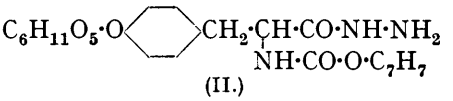
There are two main theoretical criticisms which apply to the whole of the earlier work on artificial protein derivatives prepared by the aid of the diazo-method; firstly the azo-linkage is unknown in Nature, so that the general biological validity of deductions made from experiments with compounds containing this linkage is open to some doubt; secondly the diazo-coupling not only introduces new groups into the protein but at the same time modifies the tyrosine nuclei, and such modification in itself is sufficient, as we have seen, to alter immunological specificity without regard to the nature of the group introduced. It seemed to us, therefore, to be highly desirable to devise a method of attaching new groups to protein through linkages which would at least not be foreign to Nature; more-

over in view of the importance of carbohydrate groups in natural bacterial antigens and of certain theoretical points which we had in mind we were especially desirous that the new method should include a means for the attachment of carbohydrate residues to proteins. In so far at least as simple carbohydrate residues (hexose groups) are concerned it has been possible to solve both these problems in one operation, by coupling *O*-β-glucosidotyrosine with proteins in such a way that the tyrosine carboxyl group enters into peptide combination with the free amino-groups of the protein.

The method which we devised was to bring acetobromoglucose into reaction with the ethyl ester of *N*-carbobenzyloxytyrosine in presence of quinoline and silver oxide; the resulting *O*-β-tetra-acetylglucosido-*N*-carbobenzyloxytyrosine ester (I) could on the one



(I.)



(II.)

hand be converted into the hitherto unknown β-glucosidotyrosine by deactylation, hydrolysis of the ester group, and catalytic removal of the carbobenzyloxy-residue; for the present purpose, however, it was treated with hydrazine hydrate, which effected simultaneous deacetylation and hydrazide formation; the hydrazide (II) was then converted into the azide, which could readily be coupled with proteins in alkaline solution. If desired, the carbobenzyloxy-residues could in some cases be removed reductively from the final product, but for most serological purposes this step was unnecessary. In this way protein derivatives could be prepared containing 6—12% of glucose combined in *O*-β-glucosido-linkage with tyrosine, which was itself combined in peptide linkage with the original protein.

With this method in hand we set out to study a specific problem, namely, the reasons for the non-antigenicity of gelatin and insulin. From what was already known the non-antigenicity of gelatin could be ascribed in part at least to its lack of tyrosine; no such explanation could hold, however, in the case of insulin, which actually contains an exceptionally large proportion of tyrosine; indeed the only suggestive fact about insulin in this connection appeared to be its complete lack of carbohydrate, in which it differed from most, if not all, proteins known to be antigenic; in view of the importance of carbohydrate groups among the bacterial antigens it appeared to us that this difference might be significant, both in the case of insulin and in that of gelatin, which of course is similarly lacking in carbohydrate groups. A series of antigens was therefore prepared by the method indicated, consisting of glucosidotyrosyl derivatives of gelatin, insulin and also of other proteins such as serum albumin and globulin which are themselves good antigens; animals were immunised with these derivatives individually and a detailed serological study was made.

The first point which emerged was that the glucosidotyrosyl groups introduced in the manner described were very powerfully determinant in character; the original specificity of the proteins from which they were derived was completely masked, a result which contrasts with those obtained by the earlier workers who used the diazo-method to couple glucose residues to protein; in such cases the antisera showed considerable residual precipitating power against the original protein, indicating incomplete masking of the latter. The second point is that inhibition experiments with a variety of compounds showed that the decisive determinant character of our glucosidotyrosyl groups resided in the β-glucosidophenolic linkage.

When we came to experiment with the corresponding derivatives of gelatin and insulin we first had difficulty in demonstrating that these had antigenic properties, since we were unable to obtain direct precipitation reactions between them and their antisera; we then observed, however, that their antisera would give specific precipitation with analogous derivatives of other proteins such as globulin, a fact which could only be explained by the assumption that the antisera against the gelatin and insulin derivatives contained antibodies specificially adapted to react with glucosidotyrosyl proteins; conversely we found that the glucosidotyrosyl derivatives of gelatin and insulin would precipitate

specifically with antiserum against glucosidotyrosylglobulin. These facts alone were sufficient to enable us to conclude without doubt that the derivatives of insulin and gelatin containing glucose and tyrosine were behaving as true antigens; recently, by extending the period of immunisation so as to raise the titre of the antiserum we have been successful in obtaining the final piece of evidence, namely, direct specific precipitation between gelatin and insulin derivatives and their respective homologous antisera and complement fixation with the glucosidotyrosyl-gelatin and -insulin antigen-antibody systems.

There seems therefore to be no doubt that the introduction of tyrosine and carbohydrate groups (even of such a simple character as glucose residues) has been sufficient to confer antigenicity on two otherwise non-antigenic proteins; since insulin already contains a full complement of tyrosine, it seems likely that it is the introduction of carbohydrate which is of importance, and we are indeed inclined as the result of this work to ascribe the absence of antigenic power from natural insulin to the fact that this protein lacks carbohydrate.

Our second line of effort was based on the following hypothesis. If it were possible to construct an artificial antigen in which the hapten group was a physiologically active compound, it should be also possible to prepare an antiserum against this antigen in which the antibody would be specificially adapted for combination with the compound used as hapten; such combination, by analogy with toxin-antitoxin neutralisation, would very probably mean physiological inactivation, so that the injection of such an antiserum into another animal should passively immunise that animal against the normal physiological effects of the compound used as antigen.

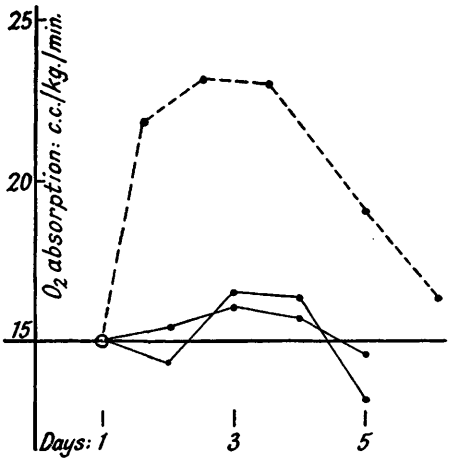
The compound selected for test of this hypothesis in the first instance was thyroxine, the choice being determined partly by my personal interest in this compound and partly by the fact that, since thyroxine is an amino-acid, we had ready to hand a convenient method for its coupling with proteins on the lines of the experiments which I have just described with glucosidotyrosine. The actual method employed was as follows. *N*-Carbobenzyloxy-3 : 5-di-iodothyronine ester was converted successively by conventional methods into the hydrazide and the azide; the latter was then coupled with proteins in alkaline solution and the product was iodinated by the action of iodine in concentrated ammoniacal solution; in this way the 3 : 5-di-iodothyronyl residues which had been introduced were converted into thyroxyl residues and at the same time the tyrosine groups of the original protein were converted into di-iodotyrosine. Such derivatives were prepared both from thyroglobulin and from horse-serum globulin and albumin, and antisera were obtained by injection of the various antigens into rabbits.

The first point of interest concerns the serological reactions. As might be expected, all the thyroxyl protein derivatives are powerful antigens and they retain little or none of the original protein specificity. In view of the powerfully determinant effect of the hapten groups introduced, it is also in accordance with expectation that a considerable degree of cross-reaction should be observed between the different antisera; thus antisera prepared against thyroxyl thyroglobulin and thyroxyl horse-serum globulin are difficult to distinguish from one another. The principal interest attaches, however, to the observation that slight but definite precipitation is obtainable between the antisera against thyroxyl thyroglobulin and thyroxyl serum globulin on the one hand and ordinary thyroglobulin on the other; this indicates that to some extent at least thyroxine acts as a hapten group in the molecule of thyroglobulin, and thus affords a finally conclusive piece of evidence that thyroxine is actually a constituent amino-acid of thyroglobulin. The supposition that all the serological reactions observed with these antigens were due to the iodine-containing groups introduced was confirmed by the results of inhibition tests; all the reactions, whether of the homologous or cross-reaction type, were more or less inhibited by 3 : 5-di-iodotyrosine alone or by thyroxine alone; they were much more completely inhibited by mixtures of di-iodotyrosine with thyroxine or with a partial degradation product obtained by enzymic digestion of thyroglobulin. This indicates that whilst the 3 : 5-di-iodophenolic grouping is important in its determinant effect, the diphenyl ether linkage of the thyroxine molecule also plays a part, a conclusion which is confirmed by the fact that antisera against the thyroxyl protein derivatives give slight

precipitin reactions with 3 : 5-di-iodothyronyl proteins which are not inhibited by di-iodotyrosine. It is noteworthy in this connection that there appears to be some analogy between the factors which influence the immunologically determinant effect of thyroxine and those which determine its physiological activity.

The serological observations which have been described were sufficiently promising to justify an exploration of the physiological possibilities implicit in the original hypothesis. Observations were therefore made on the effect of the injection of antisera against thyroxyl proteins into normal animals. No result whatever was obtained. It was then realised that this negative finding might be due to the great reserve power of the thyroid gland, which would be able to overcome any neutralising effect of the antiserum by an increased outpouring of its secretion. The experiment was therefore modified by giving the animals an intensive treatment with antiserum and following this up by administration of thyroglobulin; the metabolic response of animals treated in this way was compared with that

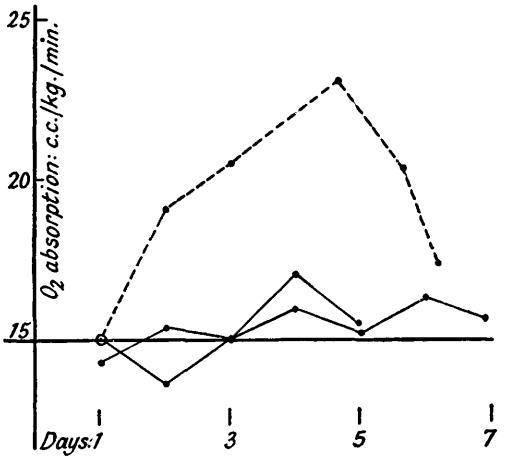
FIG. 1.



Showing suppression of action of thyroglobulin in rats by treatment with anti-thyroxyl-thyroglobulin serum. Ordinates: metabolic rate as measured by oxygen absorption. Abscissæ: time in days.

The broken line represents the effect on a rat of a dose of thyroglobulin given at Day 1 after pre-treatment with normal serum; the continuous lines show the effects of similar doses of thyroglobulin given at the same time to rats pre-treated with the antiserum.

FIG. 2.



Showing suppression of action of thyroxine in rats by treatment with anti-thyroxylthyroglobulin serum. Arrangement as in Fig. 1. At Day 1 similar doses of thyroxine were administered to the control rat pre-treated with normal serum (broken line) and to two experimental rats pre-treated with antiserum (continuous lines).

of animals which were given the same dose of thyroglobulin after a course of treatment with similar amounts of normal serum. By this technique clear-cut results were immediately obtained and it will be seen (Fig. 1) that there is no doubt that the treatment with the antiserum has induced a state of resistance or passive immunisation against the normal effects of thyroglobulin.

The experiments were then extended to the administration of thyroxine itself instead of thyroglobulin and here again a positive result was obtained (Fig. 2); preliminary treatment with antiserum produced an almost complete resistance against the effects of a dose of thyroxine which would normally increase the metabolic rate by 60—70%.

It thus appears that at least in the case of thyroxine the hypothesis upon which these experiments were based is justified. It is not however safe to deduce from the experiments with thyroxine that the theory is generally correct for the following reason; thyroxine itself is an amino-acid and it could be argued that it has to be built up into a protein or at least a high-molecular peptide before it becomes active in the body; in such an event

the successful experiments on the neutralisation of the effect of thyroxine might be no real extension of those with thyroglobulin, which themselves would fall into the class of ordinary toxin-antitoxin neutralisation.

Quite recently we have made further experiments of this type in which we have used as the physiologically active hapten group a compound which is unrelated to protein, namely, aspirin. Antigens have been prepared by coupling *O*-acetylsalicylazide with protein in alkaline solution and antisera against these complexes have been obtained in the usual manner. Inhibition reactions show that here again the immunological behaviour of the synthetic complex used as antigen is specifically determined by the new group introduced.

We have found further that animals treated with the antiserum obtained against the aspirin–protein complex show a definitely greater resistance to the antipyretic effect of aspirin than do similar animals which have been treated with the same amounts of normal serum.

I believe that these last-mentioned experiments afford strong evidence of the general validity of the theory which I have outlined above; they at least suggest the possibility of further investigations in the same lines which may be of considerable interest from the points of view of experimental pharmacology and even perhaps of practical therapeutics.