THE WASSERMANN ANTIGEN AND RELATED "ALCOHOL-SOLUBLE" ANTIGENS

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The attempt is made in this review to present material scattered rather widely in scientific periodicals—some of them not everywhere equally obtainable—and considered in textbooks and handbooks mostly from the viewpoint of its possible bearing on the sero-diagnosis of syphilis. This is explained by the circumstance that substances of the type under consideration originally came into the orbit of investigation as the antigen of the Wassermann reaction and allied methods for the diagnosis of syphilis. Subsequent immunological investigation has revealed the rather ubiquitous presence of such substances in living matter. Little, however, is known about their biological significance. It will be seen that during the past fifteen years there has accumulated a considerable amount of data which, though often loosely interconnected and sometimes awkward in chemical technic, is indicative of future developments of considerable interest. Thus, this survey is undertaken not so much from a wish of enumerating achievements as from the desire to present a basis for future investigation.

This review attempts to cover the work done since 1925. Older results are mentioned only insofar as they are thought necessary for perspective to the newer information. For older references see (1) and (2). The very numerous papers on antigens of the Forssman group are also mentioned only insofar as they are pertinent to the present problem.

In this discussion we shall avoid the collective name under which these antigens are commonly grouped, namely the term "lipid." The only thing that substances comprised under this name have in common is the readiness with which they can be extracted by organic solvents from tissue or other organized matter. In this category are substances as heterogeneous as

sterols, lecithin, cephalin, cerebrosides and the complicated phosphatides of bacteria; and it is obvious that the solubilities of some of these substances in the pure state may be of an entirely different order. Thus we think it better to forego the convenience of the term lipid in order to avoid another contribution to the lack of discrimination which this expression has furthered in the past. We prefer to use the purely descriptive, provisional term "Wassermann-type substances." It will be shown that this expression excludes the well-known major constituents present in alcoholic extracts of organized matter, such as cholesterol, phosphatides and cephalin.

Origin of present conception of the nature of the Wassermann reaction. The Wassermann reaction was originally thought to be an application of the complement-fixation method, (which was quite new at that time) to the problem of sero-diagnosis of syphilis, using as an antigen the livers of syphilitic fetuses because they contained an abundance of spirochetes (3). Two observations were soon made which shook the belief that the Wassermann reaction was directed specifically against *Treponema pallidum:* it was demonstrated that alcoholic extracts were as good or better antigens than aqueous extracts (4); second, it was found that alcoholic extracts from normal organs served just as well (4 to 6). The attempts to arrive at an understanding of this situation prior to the year 1925 will not be considered here. If the Wassermann reaction were a genuine antibody-antigen reaction at all, the observation could seemingly be explained only by envisioning an antigen of non-protein nature and derived from the host itself, thus apparently violating two principles generally accepted at the time, namely that of protein alone being antigenic and that of the "*horror autotoxicus.*" The hypothesis offered by E. Weil and H. Braun (7) contained therefore two heretical conceptions, namely that of a "lipoid antigen" derived from tissue of the host, and of an "autoantibody" directed against it[1]. A further pre-

[1] An immune precipitation with material derived from the host's body was first described by Centanni (see 7), who found in the blood of sheep infected with diastomum an antibody precipitating sheep liver extract. The development of the concept of autoantibodies cannot be discussed here.

requisite for this hypothesis is the acceptance of a special ability of *Treponema pallidum* to function as an activator of the tissue antigen. There is no direct evidence available on this matter; but differences found in the ability of "conveying" (which will be discussed in detail later on) make a conception of this kind plausible.

In 1924, Landsteiner (8, see also 9) demonstrated that specific antibody formation of the Forssman type could be evoked if alcoholic extracts containing this antigen (for instance from horse kidney) were injected in admixture with a protein (hog serum). The term hapten (10) was introduced to designate the peculiar immunological behavior of substances of this type, which react well *in vitro* but evoke antibody response only when introduced in combination with a protein.

IMMUNOLOGICAL BEHAVIOR

Experimental antibody production. The resemblance (11) of the immunological behavior of the Forssman antigen to that of the antigen contained in alcoholic organ-extracts (which react with syphilitic serum) led logically to experiments with alcoholic extracts from rabbit tissue plus hog serum (12 to 15). Abundant antibody response in rabbits was found; and the antibodies were recognized as corresponding in all essential aspects to those observed in the serum of syphilitic man and in the serum of animals infected experimentally with syphilis. Both complement-fixation and all types of flocculation reaction with alcoholic extracts were obtained.

The difference of these antibodies from those produced concomitantly against the protein conveyor is clearly defined (13) and can be confirmed by absorption tests (16). A much longer period of immunization is needed for obtaining this antibody response than is necessary for evoking antibody to the protein (13).

Conveyor. There is no indication of what actually happens in a mixture of a "lipid" hapten and serum in order to effectuate the antigenicity of the former. Landsteiner (8) thought that

the hapten might enter some kind of a loose combination with the conveying protein. Another suggestion was (12, 13) that the protein did not enter into any true chemical action with the hapten, but served only as a kind of envelope which would enable the non-protein matter to enter the cells. This was expressed under the name "Schlepper Funktion," a term which is not easily translated. The best translation seems to be conveying function, and thus what is called in German "Schlepper" will here be termed conveyor.

A third alternative explanation of the conveying function was suggested by the observation that in some cases the protein conveyor could be replaced by adsorbents like kaolin or $Al(OH)_3$. This was reported (17 to 19) to be the case for the Forssman antigen, and also for antigen contained in brain extracts. However this observation cannot be generalized because other antigens have not been found to be "completed" by adsorbents (20, 21). Experimentation with adsorbed antigens is a difficult matter because suspensions of this type are often poorly tolerated when introduced parenterally (20, 22).

It would be interesting to follow up this matter more closely. It is a situation quite peculiar for this kind of antigen, somewhat in between a full antigen and those true haptens which need actual complex-formation before being effective in provoking specific antibody formation in animals. In the case of the Forssman antigen, it is known that the necessity for a conveyor is only a relative one insofar as occasional slight antibody formation may occur with the hapten alone. There are several observations which might be interpreted as indicating a similar behavior of antigens of the Wassermann type (14).

Aqueous suspensions from syphilitic fetal liver cause the appearance of Wassermann antibodies when injected into rabbits, an effect not obtained with normal liver (23) (see below). The specific precipitate consisting of human syphilitic serum combined with antigen has been found to be an excellent immunizing agent (27 to 29). The same is true for the Forssman antigen-antibody complexes (28).

A very small amount of protein suffices for serving as a conveyor

(24). Sera of different species are effective, but they are not identical in their quality of conveying (25, 26). Horse serum is inferior to pig serum (25); human serum seems to be a good conveyor (16). Serum denatured by heating is decidedly inferior (27). Treatment of serum with diazotized atoxyl seems to destroy the conveyor function (30). Rabbit serum, either native or heated, is not a conveyor in rabbits (22, 34).

Tissue after alcoholic extraction is capable of acting as a conveyor for liver extract, but not for Forssman antigen (probably because of denaturation) (23). Bacteria are able to exercise a conveyor function (31, 32); and so does an alcoholic extract from bacteria (33).

Peptone, peptides (glycyl-lysyl-glycine) and polypeptides do not act as conveyors (35, and unpublished experiments of the author).

Lack of anaphylactic reaction. In contrast to the development of antibodies highly reactive *in vitro*, neither active nor passive anaphylaxis is obtained against antigens of the Wassermann type (36 to 38). Reports to the contrary (39) are open to objection and could not be duplicated (15, 40). It is quite possible that future investigation with purified antigens will cause modification of this statement. Floccules from syphilitic serum plus antigen do not sensitize guinea pigs to the extract, but they do sensitize to both normal and syphilitic human serum (41). (It should be noted that a hapten itself may be well qualified for causing anaphylaxis; for example, recall the reactions obtained with pneumococcal carbohydrates.)

Recent reports (32, 42) concerning passive anaphylaxis against a thermostable antigen from brain "associated with myelin and probably derived from it," may require, if this antigen is indeed linked to an antigen of the Wassermann type, some qualification of the generalization just given.

No experimental antibody production in man and animals other than the rabbit. A number of attempts have been made to duplicate in other species the antibody formation obtained in rabbits. The results have been unconvincing, and most of them entirely negative. Experiments in this direction have included

mice (43), rats (44, 45), guinea pigs (26, 27, 40, 46 to 48), and geese (49). Wassermann (50) did announce a positive result in goats, but a detailed report on this subject has never been made.

This situation remains still to be explained. It may be recalled, however, that observations not unlike this one have been recorded, for instance, the differences in antibody response in various species of animals toward pneumococcal carbohydrate. In this connection, it may be recalled also that the rabbit is generally one of the most prolific formers of circulating antibodies.

Rats and guinea pigs do not form antibody against *Trypanosoma brucei* under conditions where rabbits and humans do produce an antibody of the Wassermann type (51). Nor is antibody obtained in guinea pigs after treatment with alcoholic extract from typhoid bacilli, which does cause a response in rabbits (52, 53). A number of experiments on human beings have yielded unconvincing or negative results (54, 55). There is also no formation of the Wassermann-type antibody after (therapeutic) treatment with milk (55, 56).

It must be borne in mind that in most experiments of this kind the number of treatments was limited and that injection could not be made intravenously (57). This is important. It has been found (14, 27) that even in rabbits subcutaneous or intra-abdominal injections do not evoke an antibody response to the combination of alcoholic extract and pig serum. (Intraperitoneal injection, however, has been reported to be effective with the aqueous brain-suspension mentioned below (58) and with syphilitic liver-suspensions (23) and pig heart (59)).

In Vitro Reactions. Rabbit serum is, for reasons unknown, liable to cause non-specific deviation of complement more than that of most other species. Omission of the necessary safeguards against such occurrences has made valueless quite a number of experiments reported in the literature. However, the complement-fixation method is entirely reliable if properly conducted (3, 13, 27).

Fortunately, one is not confined ordinarily to the use of the complement-fixation test, because flocculation tests are obtained easily in most, but not all, cases. The flocculation tests are not

entirely free from the pitfalls of the complement-fixation test, but they are comparatively safer, especially under conditions where experience in the technique of complement-fixation is lacking. In any case, one of the most essential precautions in any method is the use of dilutions well out of the danger zone; but this of course makes it difficult to obtain proper information in cases where antibody titers may be low.

The range of sensitivity of complement-fixation tests and of various flocculation tests differs somewhat, and this is true not only for the lower limits of antibody titer but also for the higher ones, because inhibition by excess does occur (13, 60). It is highly desirable to use wherever possible both complement-fixation and flocculation tests together. This desirability of using more than one technique is equally important for the diagnostic application in human syphilitic infection, a point which might be stressed once more because it is too often overlooked.

The conditions for combination between antigens of the Wassermann type and the antibody have been studied quantitatively. Relatively large amounts of antigen combine with relatively small amounts of antibody (61, 62). Investigation of the mechanism of flocculation shows that antibody can account for up to 20 per cent of the total precipitate; however, much smaller amounts are sufficient for precipitation (61 to 63). It is calculated that coverage of $\frac{1}{20}$ of the surface of the antigen particles (cholesterinized heart-extract) is enough to cause flocculation. Precipitates in normal serum do not take up any protein from the serum. The isoelectric point of the antigen-antibody complex lies between pH 4.1 and 4.8 or even higher. (The isoelectric point of the antigen alone is at pH 1.8.) The critical potential of the antigen is 1 to 5 mv, and that of the antibody-antigen complex, 10 to 15 mv (63).

A separation of antigen from antibody can be effected by moderate heat (64, 65) and in concentrated NaCl solution (66); (for an older procedure see (59)). These methods are used as a means of distinguishing between specific and non-specific reactions in the practice of syphilis diagnosis: the specific floccules will

liberate antibody which can be demonstrated, while the non-specific floccules will obviously not deliver any antibody. Forssman antibody has been found to be detachable from stromata by alkali or water (67).

With purified antigens, it can be shown that one microgram suffices for complement-fixation or flocculation (68). In the inhibition tests with purified Forssman antigen still smaller amounts are found to be effective (67). However, for the stimulation of antibody response, synthetic or highly purified antigens are, as a rule, less effective than are crude extracts (69)[2].

Antibodies of this type seem to be more thermolabile than antiprotein antibodies (71, 72).

Agents fortifying in vitro reactions. One peculiarity of reactions of this type is the enhancement of the effect of the antigen *in vitro* by means of finely dispersed agents. This observation, made more than thirty years ago and put to a practical application of considerable importance (see 1), is a purely empirical one; and it has contributed its part to the confusion concerning the nature of the test for syphilis. Cholesterol was found to be especially convenient for this purpose and has maintained its position as a fortifying agent ever since. It was demonstrated that its peculiar qualification is based on the formation of microscopic crystals when it is added to aqueous solutions. These crystals furnish the surfaces on which the antigen proper is adsorbed and on which the antigen-antibody reaction takes place (61 to 63). It is, however, not yet known whether this adsorption contributes materially to the ease of combination or whether it is merely an adjuvant to aggregation and the formation of visible particles. This latter action certainly plays an important role in flocculation; however, the effect is just as marked in complement-fixation, and there the problem of this effect remains shrouded in the veil of our lack of knowledge of the action and mechanism of complement-fixation generally. Recent investigations suggest once more a definite optimal ratio of cholesterol (73).

[2] In the case of purified Forssman antigen large amounts (100 mgm. per dose) were found to be less effective for eliciting antibody formation than were small doses (1 mgm.) (70).

Cholesterol is by no means the only substance active in this way (74). Cholesterol derivatives are found to be quite similarly active if used in proper quantities (75). Commercial lecithin is another agent of this kind (76); (commercial lecithin preparations may, however, contain considerable amounts of cholesterol.) Other examples of non-specific fortifying agents are cited in (77).

Special mention should be made in this connection of the effect of phenol (78, 79). It may be that this effect is similar to that of cholesterol, a physical one, effectuated by the creation of fine protein precipitates. It is, however, possible that the effect is a chemical one in the sense that phenol may break up the combination between tissue protein and antigen. There are no direct chemical observations on this point. "Unspecific" reactions caused by phenol-alcohol have been suggested as due to the liberation of antigen from serum (80).

A similar mechanism may be responsible for the formation of antibody of the Wassermann type observed in the sera of rabbits immunized with diazotized atoxyl-protein or metanilic acid-protein (30).

OCCURRENCE AND BIOLOGICAL ASPECTS OF WASSERMANN-TYPE ANTIGENS

Ubiquity of the Wassermann antigen. Materials reacting with syphilitic sera have been found to be present in practically all living matter, especially in organs of all kinds of animals (1, 81). Alcoholic extracts of microörganisms contain the active principle quite frequently (see table 1) and so do alcoholic extracts from plants (82, 83).

However, this agent is not the only alcohol-soluble antigen present in living matter. The alcohol with which it is commonly extracted quite often extracts other substances of a more specific nature. Thus it was found (84) that Forssman and Wassermann antigens are present together in alcoholic extracts of organs from animals of the heterophile group.[3] The two antigens can be separated by specific absorption (47).

[3] For this reason, it is inadvisable to use tissue of animals belonging to the Forssman group for the preparation of extracts for the serodiagnosis of syphilis (85).

Antigens with organ-specificity. Alcoholic extracts of rabbit hearts, livers, and kidneys were found to react preponderantly with sera obtained by the immunization with the respective organ extract (plus hog serum) and only to a lesser degree with extracts of different organs (13, 86, 87). This situation is more marked in the case of extracts from brain ("neurohapten") (26, 45, 88 to 90). Immunological differentiation of grey and white substance has been demonstrated (91). Brain specificity appears in the 3rd to 4th month of human embryonic development (91, 105). Antigens of considerable organ-specificity can be found in alcoholic extracts of blood cells, testis (92), ovary (93), thyroid (94), hypophysis (95), and so on (49, 96 to 101). Leucocytes also contain a highly specific antigen (103, 104). The degree of specificity varies not only from organ to organ, but also within each individual immune serum. Cross reactions are frequent, and they indicate relations that can be more or less clearly demonstrated by absorption tests. A specially pointed example of this is the cross reaction between brain and testis (92, 102) which appear to have in common an antigenically rather active material; the same antigen has been recently found to be present in alcoholic extracts of corpus luteum (but not in other ovarian tissue) (225). It would be of interest to know whether the antigen common to brain, testis, and corpus luteum is identical with that causing the cross reaction between brain extracts and spirochetes (100).

Both older and more recent findings (42, 58, 88, 89) suggest that an organ-specific antigen may be obtained from brain by autolysis or by the action of microörganisms. It is described as strictly tissue-specific and not species-specific, heat stable, non-dialyzable, and linked to myelin for chemical reasons as well as from the fact that it is not found in fetal brain; more recently, however, it has been suggested that it may be rather of protein nature (32). It is unknown at present how this water-soluble antigen is related to the material contained in alcoholic extracts[3a].

[3a] In a paper which came out when this review was already in press, the existence of two different heat-stable antigens in brain tissue has been demonstrated. One of them is soluble in alcohol and, presumably, is the one which is active in

This antigen, associated with the white substance of the brain, is also present in the nerves. It presents the same antigenic relationship to testis as was pointed out for alcoholic brain-extract, and thus it is conceivable that the water-soluble antigen may be the bearer of this relationship.

Injections of milk (55, 116) and of yolk (117) have been found to cause the formation of antibodies of the Wassermann type in rabbits; in these cases no conveyor is necessary.

Antigen with tumor-specificity. Malignant tumors contain more or less regularly an agent which goes over into alcoholic extracts and which is common to most kinds of malignant growth (106 to 111). An antibody against this tumor-specific agent is found not only in the serum of animals immunized with tumor extracts, but also in a considerable percentage of human beings affected with malignant growth (112). It remains to be seen whether this tumor-specific antigen from alcoholic extracts is identical with that found in "heavy material" sedimented by centrifugation at 27,000 rpm from aqueous extracts together with Wassermann, organ-specific and Forssman antigens (222). "However as matters stand now this antibody is not found frequently enough, and especially not early enough in the course of the disease in order to be of diagnostic importance" (113, see also 114). There is also found a rather high percentage of "unspecific" reactions (114, 115), explained as "Zerfallsspecifität" (106).

Antigens from animal parasites and bacteria. Specific alcohol-soluble antigens have been obtained from tapeworms (118, 119), hydatid fluid (120) and bilharzia (121). In bacterial extracts the situation can be highly complicated. Specific antigens, antigens common to large groups of bacteria, and antigens of the Wassermann or the Forssman type have been found; and the various antigens are often present together in the same extract. This situation is presented in table 1, which lists the antigens which have been found in alcoholic extracts, either alone or in company. The blank spaces mean that no data have been re-

alcoholic brain extracts. The alcohol-soluble and the alcohol-insoluble antigens could be differentiated by immunological methods. Henle, W., Chambers, L. A., and Groupe, V. 1941 J. Exptl. Med., **74**, 495.

ported. All of the reports refer to the antigen response in rabbits. The injection of alcoholic extracts from typhoid bacilli into guinea pigs apparently does not lead to antibody formation (52, 53).

Antigens from spirochetes and trypanosomes. Of special interest are two types of microörganisms, namely *Treponema pallidum* and the trypanosomes (51, 134, 135). The behavior of the former will be discussed in detail later on. Trypanosomes are

TABLE 1

Antigens to be found in alcoholic extracts of bacteria

ALCOHOLIC EXTRACTS FROM	HOMOLOGOUS ANTIBODY FORMATION IN RABBITS	CROSS REACTIONS			REFERENCE
		Bacterial	"Wassermann" (tissue extract)	Forssman	
Proteus X_{19}	+		+	+	33, 122, 123
Typhoid bacilli	+				122, 124
Paratyphoid A and B bacilli	+				122, 124
Dysentery bacilli, Shiga, Flexner	+			+	122, 124, 125
Tubercle bacilli	+		+		123, 126 to 128
Diphtheria bacilli	+	Numerous (*a*)			129, 130
Meningococci	+	None			131
Pseudomonas aeruginosa.	+			−	132
Streptococci	+	Group-specific			133
Streptothrix sp.	+				129
Spirochetes	+		+	−	See text
Trypanosomes	+	(*b*)	+	−	51, 134, 135

(*a*) Streptococci, staphylococci, colon, tubercle and pseudodiphtheria bacilli, *Proteus* X_{19}, and *Bacillus subtilis.*

(*b*) Negative for *Treponema pallidum.*

evidently rich in the Wassermann-type antigen (134, 135); but their antisera do not react with spirochetal suspensions (51).

Alcoholic extracts of microörganisms differ, as a rule, in one essential point of behavior from alcoholic extracts of organs: they are fully antigenic. This means that they evoke antibody formation if introduced in the animal body alone, without the presence of a conveyor, and this is also the case with spirochetal extracts. It is thus necessary to avoid bacterial contamination when such antigens are used for immunization.

The role of bacteria and vaccine virus used recently in connection with the preparation of antisera to brain emulsion still remains to be determined (42, 58).[4]

Availability of antigens. In the representative case of antibody formation against antigens of the Wassermann type, alcoholic extracts in combination with a conveyor are used. The employment of suspensions or aqueous extracts of an organ will not cause antibody formation against the Wassermann-type antigen which is present in alcoholic extracts, but merely against species-specific (and often also organ-specific) substances, probably proteins. The same is true of sediments obtained from saline tissue-extract by high-speed centrifugation (222). Furth and Kabat conclude that the Wassermann antigen is contained in a large complex molecule. In any case, it appears that these peculiar antigens are usually not available in the cell in a form that is antigenically effective, as if such antigens were masked. A quite instructive case of this type is the behavior of blood serum (137). Its introduction into a rabbit will not cause the formation of antibodies that react with alcoholic extracts. However, an alcoholic extract of the serum injected together with the whole serum does evoke antibody formation of the Wassermann type (see 138). This observation may help to an understanding of why a substance in the body can be iso-antigenic. It is also an important argument in explaining why substances in the diseased body (substances that are not antigenic under normal circumstances) may become antigenic. In such a case, antigenicity might appear if in *infected* tissue a breakdown occurred that liberated the antigen.

Not all substances contained in alcoholic extracts are as completely masked as in the example given above. Antibodies against antigens extractable by alcohol are evoked by immunization with aqueous extracts from organs containing Forssman antigen, and also from such extracts containing the related blood-group A antigen. The same is true for the antigen characteristic of malignant growth and for several bacterial antigens.

[4] According to data published since this paper was finished, bacteria can be replaced by kaolin and other adsorbents. Their role seems, therefore, to be merely a physical one (136).

The similar cases of syphilitic fetal liver and of Wassermann-type antigen in milk and yolk have already been mentioned.

Competition of antigens. The term "masked" antigen has been used, however, in another quite different sense, namely for the suppression of one antigen in a mixture of several present in an extract, suppression either of the antibody response, or of the antigen's *in vitro* reactivity, or of both. Observations of this type have led to the hypothesis of the "competition of antigens." This notion is certainly incorrect as a generalization; but phenomena of this pattern are realities just the same (139 to 143). The basis of most of them is probably a difference in antigenicity or ease of combination. In certain instances, chemical causes for this phenomenon have been suggested: differences of solubility (144), formation of "micelles of antigens" (loose compound-formation) (145), and on the other hand, the breakdown of compounds (100).

Lack of protective properties. Besides the antibody characteristic for the serum of man infected with syphilis, antibodies of this type seem to be evoked by tuberculous infection (146), and, less regularly, by malignant growths (see above). None of these antibodies seem to be associated with a protective mechanism. This includes syphilis (147, 148). Contrary data lack confirmation; and recent evidence for some protective quality in syphilitic sera does not prove that the protection is due to the Wassermann antibody (29, 71).

The diseases just mentioned have in common the peculiarity that an effective resistance is not built up or is not of a predominantly humoral type. The peculiar antigenic inertia of tissue spirochetes has been a matter of comment (149).

"Biological false reactions" and their theoretical significance. The sero-diagnosis of syphilis has always involved precautions against the occurrence of non-specific positive reactions. Technical progress has diminished this difficulty, but this problem needs to be discussed here because of its theoretical significance. Non-specific reactions have been attributed in the past to what is called "increase of lability" in certain sera, but there has never been a satisfactory explanation of what is meant by the term.

Increase of the globulin content of such sera (frequently considered in the past to be the cause) has obviously nothing to do with the phenomenon, as there is indeed no correlation between the globulin content of the sera and their ability to react with alcoholic tissue-extracts (see for instance 150, 151).

More recently another explanation has found its expression in the term "biological false reactions." This expression implies that non-specific reactions might be caused by the presence in the serum of antibodies of related qualities which cause cross reactions because of similarities in their antigenic counterparts. It would be unique indeed, if the production of antibodies of the type of the Wassermann antibody were entirely restricted to syphilitic infection. As it is, it is peculiar enough that only in syphilis is this antibody response strongly and regularly manifested, at least in man. It is, however, becoming increasingly clear that a fundamentally similar antibody formation can be engendered by various other disturbances of human physiological equilibrium, and it is necessary to take full cognizance of this situation in order to arrive at a deeper understanding of the problem at hand.

Reports on "biological false reactions" have specified a number of infectious diseases in which such observations (152, 153) have been made: malaria, leprosy, streptococcal endocarditis (154), measles (155), infectious mononucleosis (46), diphtheria (52), and even after smallpox vaccination (223). Yaws and pinta (or cavate) (219) are omitted from this enumeration because of their peculiar relation to syphilis, which makes it likely that immunological relations are very close (153). Recurrent fever, another human spirochetal disease, does not lead to a positive Wassermann reaction with any degree of regularity. The occurrence of antibodies of similar type in tumoral diseases and their relation to embryonic growth have been discussed above, and it is only necessary to recall these facts in order to understand that "biological false reactions" in pregnancy and tumoral diseases belong in the same category.

It may well be that one or another item of the incomplete list given above is included as a result of imperfect technique, just

as a much larger number has been eliminated for similar reasons in the past; but it has become increasingly certain that "biological false reactions" are not entirely to be eliminated by improvement of method. Moreover, such false reactions cannot be eliminated by confirmation reactions based upon cleavage of the antibody-antigen complex (65, 66).

Fortunately these "biological false reactions" are of low titer as a rule. Moreover, their interference with specific reactions in diagnostic tests for syphilis can be greatly diminished by modifying the technique in such a way that small amounts of antibody do not react with the antigen. Unavoidably, this increase in specificity has to be paid for by elimination of low-titer syphilitic reactions. The gain in safety thus achieved represents the most significant advance in the technique of the serological diagnosis of syphilis, as a highly competent observer has remarked (152).

"Normal" antibody of the Wassermann type. In connection with the problem of false reactions, it might be mentioned that complement-fixing antibodies against cholesterol have been reported to occur in several human diseases (156) and that complement-fixation with lecithin has been seen frequently in monkey sera (157). One wonders whether the so-called nonspecific reactions of rabbit sera (see above) may not some time find a similar explanation. Positive Wassermann and flocculation reactions have been recently reported in a considerable percentage of sheep (158) and beef (224) sera.

Role of tissue-specific antibodies in pathology. The finding of organ-specific antibodies has aroused speculation as to their possible bearing in pathology. The recent report (159) on kidney antibodies and their relation to nephritis is one example; another is the possible relation of syphilitic brain diseases to brain-specific antibodies (160). However there is no experimental evidence as to the latter. In view of the pronounced organ-specificity of the Wassermann-type antigen from the brain, brain-specific antibodies have been expected to be found in the spinal fluid of patients with syphilis of the central nervous system. However, reports of such findings could not be confirmed (46, 161, 162); and the reasons for this failure are unknown. There is no suggestion of damage to the brain from the experimental introduction

of Wassermann-type, anti-brain antibody (46, 163). There is also no evidence as to the role played by tumor-specific antibodies (164). In this matter there might enter the old observation (165) that there are special mechanisms which tend to minimize the effect of complement upon the tissue (red blood cells) of the same species.

THEORY OF SPIROCHETAL ORIGIN OF WASSERMANN ANTIGEN

Basic observations. Wassermann's original conception that the antibody in syphilitic sera is a truly antispirochetal substance was abandoned by him after a bitter struggle. It experienced, however, an unexpected resurrection when it was found that alcoholic spirochetal extracts are antigenic and are suitable antigens for practical diagnostic purposes. It will be readily acknowledged that the idea possesses the advantage of simplicity, when presented in its modernized form which recognizes that the antibody is certainly not of the type commonly encountered in infectious diseases. The experimental evidence for this hypothesis rests in the main on the observation of the formation of Wassermann-type antibody in rabbits and in man, after injection of spirochetal antigen (23, 46, 162, 166 to 168). However, this evidence is considerably less convincing than appears at first sight.

Culture of spirochetes. The most competent students of the biology of *Treponema pallidum* do not feel that cultivation of this microörganism in the test tube has ever been actually achieved (169, 170). The decision is a difficult one, because whatever it is that has been cultivated, infectivity for the rabbit and monkey is lost at once. Spirochetes morphologically very similar to *Treponema pallidum* occur under conditions which make it appear quite possible that they may have been substituted during transfer from the syphilitic lesion. Certainly, those spirochetes that are in use in Germany for the production of vaccines for experimental purposes and sold commercially as a diagnostic antigen are not likely to be *Treponema pallidum* (166, 169, 171).[5] This in itself may not be objectionable, because it is quite possible

[5] Culture spirochetes seem to be antigenically homogeneous (189) with the possible exception of one strain (190).

that the antibody formed in the case of immunization with spirochetal antigens is group-specific rather than specific for *Treponema pallidum* (168, 174).

Objections to the available evidence. Whatever they may be, the spirochetes that are used as above have to be cultivated in media containing serum with or without tissue. Suspensions made from such media may very well carry antigenic material derived from the medium, and not from the microörganisms (57, 172), in sufficient quantities for eliciting antibody formation. Even washing the suspension, if done, does not preclude the retention of small amounts of antigenic matter from the medium.

There were, of course, controls made with the medium alone (173) or media inoculated with bacteria (174), for instance colon bacilli, which did not lead to formation of the antibody in question. But these controls cannot be relied on for two reasons: (*a*) the uninoculated medium was not subjected to the metabolic action of the spirochetes, which may quite conceivably lead to the liberation of originally masked antigen (57), just as the alcoholic extraction does in the case of the serum referred to above (61). (*b*) Moreover, media inoculated with bacteria may not be subjected to the same kind of breakdown that is caused by the spirochetes (57). It cannot be overlooked in this connection that the conception of the Wassermann reaction as caused by an antigen derived from the body necessarily postulates an action of the spirochetes upon the cells of the body different from that caused by other pathogenic microörganisms. This is implied in the very fact that the Wassermann reaction is characteristic for syphilis and no other disease. (The admission of the possibility of Wassermann-type antibody formation by other causes would modify this statement only in the sense of a difference in degree and not in kind.)

It must be pointed out that many of the spirochetal antigens used in experimental work contain phenol. The importance of this compound for the practical value of the antigen was stressed by the originator of a widely used preparation (173, 175). It was mentioned before, that phenol acts not only as a fortifying agent (*Verstärker*), but it is supposed to set free the masked antigens.

Thus, the value of all experiments using phenolized antigens for proving the spirochetal nature of the Wassermann reaction is doubtful.

Differentiation of spirochetal and tissue antigens. There is suggestive evidence that the Wassermann reaction and spirochetal complement-fixation occur independently (97, 176, 177). This is not necessarily proof of a complete difference in the antibodies, because similar observations can be made with any two Wassermann extracts. Variations in the content of antigen and in its chemical properties could well account for differences. Differentiation of the spirochetal and the Wassermann antibody by means of absorption has been repeatedly claimed (166); but others have not succeeded (168, 178).

There is a conflict of evidence on one important phase of this problem. According to some observers, sera obtained by immunization with both spirochetal extracts and spirochetal vaccines can react to a similar degree with both alcoholic spirochetal extracts and alcoholic tissue-extracts (179, 180). Others, and they are in the majority, with the best controlled experiments, found spirochetal antisera to react solely or quite predominantly with spirochetal extracts (43, 46, 87, 100, 116, 168, 181, 182). This latter is the case especially in the reports concerning human beings treated with spirochetal vaccines for the double purpose of non-specific (fever) therapy and experimentation on antibody formation (167, 179, 183, 184). The same relation has been reported for skin reactions in human beings (183).

Cross reactions with brain-extracts have been described for such cases where immunization was effected by mixtures of spirochetal extracts and hog serum (100, 162). It has been suggested that this might have been due to the presence of cholesterol in both antigens. Spirochetal extracts are markedly increased in their reactivity *in vitro* by cholesterol (100).

Trypanosomal Wassermann reactions. Reactions clearly of the Wassermann type can be obtained from both animals and man immunized with trypanosomes (51, 135). This, however, remains an interesting parallelism with no conclusive value for the problem of the spirochetal origin of the Wassermann reaction. Anti-

trypanosomal sera, as mentioned before, do not react with spirochetal antigens (51).

There is another fact that should be kept in mind; spirocheticidal effects, so readily obtained after experimental immunization, have not been observed in the case of actual infection either of man or of experimental animals (185).

The choice between the theory of tissue origin and that of spirochetal origin of the Wassermann antigen appears to depend on deduction rather than on direct evidence. In view of the data presented above on the ubiquitous occurrence of the Wassermann antigen and its congeners, it would not be surprising to see the Wassermann antigen isolated both from tissue and from spirochetes. In this case, a decision between the two competing theories could, of course, not be made with chemical methods, and it is difficult to see how it could be made by an immunological approach.

It should be stressed that the uncertainty concerning the origin takes nothing away from the established facts on the immunological and chemical peculiarities of the Wassermann antigen.

CHEMISTRY OF ANTIGENS OF THE WASSERMANN TYPE

Haptens of known composition. Before entering the problem of the chemical nature of Wassermann-type antigens, brief mention should be made of the substances other than proteins that have been found to possess antigenicity. The best known examples are the polysaccharidic substances isolated from bacteria or other cellular sources. So far as these substances are composed solely of sugars and amino sugars, they possess no, or only incomplete, ability to evoke antibody formation; they are, however, reactive *in vitro*. Full antigenicity is found, however, in those cases where polysaccharides occur as complexes with fatty acids, and phosphorus- and nitrogen-containing compounds as is the case in gram-negative bacteria of the coli-dysentery-salmonella group, (*antigène complet*). It is likely that the phosphatides isolated from tubercle bacilli are chemically constituted in a similar fashion. They are also fully antigenic. The component which determines the specificity is in either case the polysaccharide complex. The "lipid" moiety is not antigenic.

Relatively simply constructed substances have been found to be antigenic in the sense of being haptens which need a conveyor for eliciting antibody formation. They are by this property linked to the group of the Wassermann-type antigens.

The first reports on the hapten quality of such substances concerned lecithin and cholesterol (186). These experiments were, however, made on commercial preparations of low purity so that there remained doubt as to whether the antibodies observed were really against cholesterol or lecithin[6]. Whereas the reports concerning the antigenicity of commercial products (187, 188, 192)[7] and similar preparations of cephalin and cerebrosides (188) have been confirmed, purified lecithins and cephalins from egg and brain have been found to be non-antigenic (187, 193 to 195), and lecithin from soya beans of doubtful antigenicity (195). On the other hand, antibody formation was obtained against a synthetic lecithin, the tristearyl-lecithin synthesized by Gruen (196, 197), and against recrystallized cholesterol and several congeners (di-hydrocholesterol, cholesterin oxyde, ergosterol (191, 196, 198)). Objections (199) to the validity of these observations from the point of view of the small amount of complement fixed are the less valid, because cholesterol and its derivatives do not only give complement-fixation of high specificity (196, 198), but also are flocculated specifically by their corresponding antisera (200). It has been found that *not* all cholesterol derivatives have hapten quality, (191, 201): oxycholesterol, cholesteryl bromide and several cholesterol esters seem to be devoid of this property. Highly purified cerebron and lignoceryl-sphingosine are non-antigenic, whereas a polydiaminophosphatide from spleen, lung and liver has been shown to be a highly effective and specifically reacting hapten (202).

Quantitative considerations (69) show that the synthetic antigens are both less antigenic *in vivo* (as measured by the

[6] Low purity of testing material might explain in part the observation of odd cross reactions between lecithin and cholesterol and the respective antisera (186). However, similar cross reactions have been observed with lecithin free of cholesterol admixture, for which no explanation has yet been offered (191).

[7] Ovotellin, a full antigen, has been separated from crude egg lecithin (191). However, no effort has been made to determine the true relation of this antigen to lecithin antisera by the absorption test.

amount of material and the duration of treatment necessary to evoke antibody) and *in vitro* (as measured by the minimal effective amount) than are natural antigens of the Wassermann type (see above).

These observations tend to show that substances of well-known constitution are able to act as antigens in a way similar to those observed in natural ones of the Wassermann type. They increase the range of materials in which to look for substances of hapten nature. They make us aware that substances of this type may be on the borderline between haptens and non-haptens, so that small chemical differences may decide whether they can produce specific antibodies or not. In addition, they open an interesting field for the study of specificity and other problems of immunochemistry, until now little explored.

Chemistry of tissue antigens. There remains to be discussed what little is known about the chemistry of the antigens of the Wassermann type. The active substance represents only a small fraction of the total extracted by alcohol. The antigen is carried down from alcoholic extracts by cadmium chloride (203, 204). (Precipitates obtained (204) in this way are still used for diagnostic purposes.) However, the conclusion that the Wassermann antigen is a lecithin, has not been borne out by further investigation (220). It was also realized early that the antigen is insoluble in acetone (204). It is a question whether the substance is really alcohol-soluble, because it has been demonstrated (205) that both the Wassermann and the Forssman antigens can be adsorbed from alcoholic solution by kaolin and other inorganic adsorbents and eluted into water or saline to give clear solutions (20, 206)[8]. It may be that the alcohol-solubility at primary extraction is due to co-solubility caused by the presence of other substances; but this remains to be proved.

The Wassermann antigen is recently reported (222) to be sedimented from saline tissue-extracts by high-speed centrifugation (27,000 rpm. for one hour) together with organ-specific antigens, Forssman antigen, and several enzymes. However, injection of

[8] Elution is also possible in organic solvents like benzene (209). Further data on adsorption and elution are given in references 206, 209 to 211.

these sediments into rabbits does not cause formation of antibodies of the Wassermann type, whereas other antibodies, e.g., that of the Forssman type, *are* evoked. A logical interpretation of this observation would be that in these sediments (and presumably also in the intact tissue) the Wassermann antigen is hidden within large complexes and that for this reason its antigenic activity is not exerted (i.e., masked, see page 305). Alcohol extraction would then be needed in order to liberate the Wassermann antigen from its connection with other material and make it effective as an antigen. If such a conception is true, a similar effect as that of the alcohol could be surmised as becoming effective in diseased tissue. Such a conception would be valid regardless of whether the complex from which the antigen is liberated is derived from the cells of the body, from spirochetes, or from both.

A combination of the methods of acetone precipitation and $CdCl_2$ precipitation results in preparations that are effective in amounts of the order of one microgram (68, 207). Further elimination of ballast substances can be achieved by adsorption followed by elution into boiling methyl alcohol (208). Preparations obtained by the $CdCl_2$-acetone method have been found (68) to be non-reactive if tested immediately, but fully reactive after standing at room temperature for a few days[9]. These purified antigens are not dialyzable (214); and hydrolysis destroys their activity (68, 215, 216). Analytical data on the purest preparations obtained up to now are given in table 2.

All of these materials are not only reactive in complement-fixation and in flocculation with both rabbit and human (syphilitic) sera (68, 213), but they retain their full hapten quality of evoking antibody formation (213). They retain, so far as is known, their partial or total organ-specificity. Work along similar lines has been done with alcoholic extracts from brain (214) and from tumor (216).

Another method (217) of purification takes advantage of the solubility of the antigen in acidified 80 per cent alcohol when shaken together with petroleum ether. By a combination of this

[9] Is this an oxidative effect? There are other indications of the influence of oxygen on alcoholic tissue extracts (205, 212).

method with adsorption and elution, preparations have been reported with a purification factor of 100; but analytical data on these are not available as yet. Further details on this phase of the subject cannot be given without a disproportionate amount of discussion beyond the limits set for this review.

Recently, the non-protein part from floccules of syphilitic serum and cholesterinized tissue extract (Kahn) has been analyzed (221). The results, so far as they go, fit well with the data given above.

A protagon from the brain has been isolated which contains 2.4 per cent N and 0.5 per cent P and which can function as a

TABLE 2

Analytical data on the purest preparations hitherto obtained

ANTIGEN	NITROGEN	PHOSPHORUS	SULFUR	FEHLING (CARBOHYDRATE)	FATTY ACIDS	REFERENCE
Heart.....	Little	+	0	0[a]		(68)
Brain.....	1-2%	0		2%[b]	+	(214)
Tumor.....	0	0		Very little	+[c]	(216)
Forssman....	2-2.3%[d]	0.1%[e]	0.17%[e]	30%[f]	Probably +	(67)

[a] 7% by reduction of potassium ferricyanide (214).
[b] Probably 50% as creatinine.
[c] Cholesterol ester +.
[d] ⅓ or more as hexosamine.
[e] From contaminants ?
[f] Aldohexose. Total C = 60 to 70%.

hapten (218). Its antiserum has been found to give cross reactions with aqueous but not with alcoholic extracts from brain, and with lecithin prepared from brain. This serum did not react with alcoholic extracts from other tissues, nor with lecithin prepared from egg, nor with cerebron or sphingomyelin.

The method of tryptic digestion and alcohol fractionation and the trichloracetic acid method, both successfully employed for the isolation of the "antigène glycido-lipidique" of gram-negative bacteria, have not proved effective with spirochetes (168).

It is difficult to draw any conclusions as to the nature of the effective material from the data available at the present time.

Nor do they give any additional indication as to whether the antigen which causes the formation of the Wassermann antibody in syphilitic man is furnished by the spirochete or by the host.

CONCLUSION

Owing to the scantiness of biological and especially of chemical data the immunological aspect necessarily is predominant in this review.

Syphilitic infection of man and animals causes a production of antibodies directed against one or more closely related substances of a peculiar kind. Immunological experimentation has further substantiated the antigenic qualities of these substances. The mere fact of their rather ubiquitous distribution should arouse general biological interest which has so far taken cognizance of the Wassermann antigen only in a perfunctory way.

The observations derived from and concomitant to experimentation concerning the Wassermann reaction have led to the detection of substances of similar immunological behavior but differing from the Wassermann antigen in more pronounced specificities and in being more limited in distribution. The existence of chemo-specific and organ-specific antigens has been established. The investigation of the significance of these antigens (and their corresponding antibodies) is only in its beginning.

We are faced with a maze of interrelated substances which are carriers of complicated and subtle specificities. This explains why their serological differentiation is often beset with difficulties. The simultaneous occurrence of multiple specificities within one tissue or one kind of cell and the corresponding formation of multiple antibodies upon immunization have been found to be frequent. The degree of antigenic predominance probably depends not only on quantitative relations, but also on factors such as solubility, adsorption, and complex formation which are all hidden behind the term availability. The determination of all of these factors will be a necessary part of future work in this field.

Similarities in immunological behavior form the common link of this group of substances. They are all extractable by organic

solvents from tissue and other organized matter.[10] They have been designated in this review as Wassermann-type antigens. Nobody is more aware than the author of the preliminary and provisional nature of this classification and its need for verification and rectification by chemical methods. In taking stock of the present state of our knowledge in this matter, it is hoped that attention will be drawn to a field invitingly open for further investigation.

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Note.—This review covers publications available up to September 25, 1941. Citation of the great number of communications on the many experimental details is not permissible owing to limitations of space. In many cases, the selection of references is made not so much from the standpoint of priority of publication as for giving access to sources of details. Where the work of one laboratory is reported in a series of communications, the most recent publication containing references to prior work is given preference for quotation.

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