AN IMMUNOLOGICAL STUDY INVOLVING CARCINOGENIC HYDROCARBONS

By

Evelyn Lenore Oginsky

Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

1946

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INTRODUCTION

Although the ultimate cause of spontaneous tumors* in man and animals remains unknown, it has been demonstrated that tumors can be induced experimentally by viruses (e.g. Reus sarcoma, Shope papilloma, and Bittner milk-factor tumor), and by chemical compounds of known structure. The chemicals are mainly of two types, namely, hydrocarbons containing a 1,2-benzanthracene nucleus, and derivatives of azobenzene, e.g. butter yellow.

One of the hydrocarbons, 3,4-benzpyrene, apparently was responsible for occupational tumors among early workers in the coal-tar industry. The hydrocarbons, however, have never been directly implicated in spontaneous cancer. The chemical structure of these hydrocarbons is somewhat similar to the bile acids, sterols, and sex harmones and it is possible that carcinogenic (tumor-producing) compounds may arise in the body by transformation of these normal metabolic substances or their precursors.

In accordance with this idea, and since it should be possible to immunize animals against experimental tumors due to hydrocarbons, it might be possible to immunize animals against spontaneous tumors. Such an immunization might have to be directed against carcinogenic hydrocarbons similar to those known at the present time or against active hydrocarbons or derivatives as yet unknown.

* Spontaneous tumors are uncontrolled new growths arising without any apparent external cause. Cancers are malignant tumors which invade and destroy tissues.

It became of interest to develop a method of producing antibodies which would react not only with the hydrocarbon involved in the immunization procedure, but with other hydrocarbons as well. It is with the production and study of such antibodies that the following thesis is concerned.

HISTORICAL

A search for chemical agents active in carcinogenesis was begun after the discovery by Yamagiwa and Ichikawa (54, 55) in 1915 that skin tumors could be produced on the ears of rabbits by prolonged painting with coal tar. It was found by Kennaway (31) that heating acetylene or isoprene in an atmosphere of hydrogen resulted in the formation of carcinogenic hydrocarbons active against mice. Hieger (26) determined that the fluorescence spectra of these and other carcinogenic mixtures, such as aluminum-chloride treated tetralin (32), heat-treated cholesterol (33), gas works tar, and shale oil, were characteristically the same. On comparison with the fluorescence spectra of known hydrocarbons, he found that the spectra of the carcinogenic mixtures were remarkably similar to that of 1,2-benzanthracene. This indicated that the carcinogenic hydrocarbons were of the polycyclic aromatic type and possibly derivatives of 1,2-benzanthracene.

About thirty-five of such derivatives were synthesized by Cook, Kennaway, and their colleagues at the Royal Cancer Hospital in London (10, 6, 2). The skin in the interscapular region of mice was painted several times weekly with a benzene solution of the hydrocarbon under test. The first synthetic compound which proved carcinogenic under these conditions, and caused the formation of malignant tumors at the site of application, was 1,2,5,6-dibenzanthracene.

These English workers isolated from coal tar distillate, and later synthesized, a very active carcinogen, 3,4-benzpyrene (9), which has the typical fluorescence spectrum of the other carcinogenic

agents. It is the only potent carcinogen which thus far has been isolated from coal tar (8). Its chemical structure is shown in Fig. 1.

Still another hydrocarbon, of similar structure, 20-methylcholanthrene, (see Fig. 2) was prepared by Wieland and Dane (53) by dehydrogenation of dehydronorcholene, a compound which had previously been obtained by Wieland and Schlichting (52) from desoxycholic acid. Cook and Haslewood (7) confirmed Wieland's results on the formation of 20-methylcholanthrene from desoxycholic acid, and found that it was carcinogenic. They found it to be a more rapid acting carcinogen in mice than any previously tested, as it caused tumors in seventy days, as compared with two hundred and ten days for 1,2,5,6-dibenzanthracene and one hundred days for 3,4-benzpyrene. 20-Methylcholanthrene also has been prepared by the transformation of cholic acid and by actual synthesis by Fieser, Newman, and Seligman of Harvard University (19, 20). Both cholic acid and desoxycholic acid are found in the bile of animals, where they occur as parts of the bile salts involved in fat metabolism.

Several hundred compounds, similar to those described above, have been synthesized and tested for carcinogenic potency (23). The experimental work described in this thesis, however, is concerned primarily with 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene. It would be well to point out at this time that systematic studies of the derivatives of 1,2-benzanthracene, conducted by Fieser, Shear, and their colleagues (18), indicated that carcinogenic activity was associated principally with derivatives of the following general nature. Carcinogenic potency was found to be dependent on the structure and the position of the substituents in the 1,2-benzanthracene nucleus, which

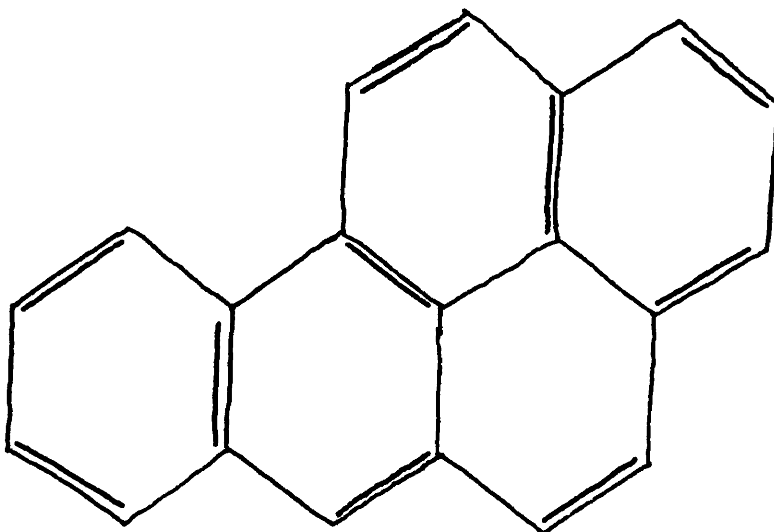


Fig. 1 3,4-BENZPYRENE

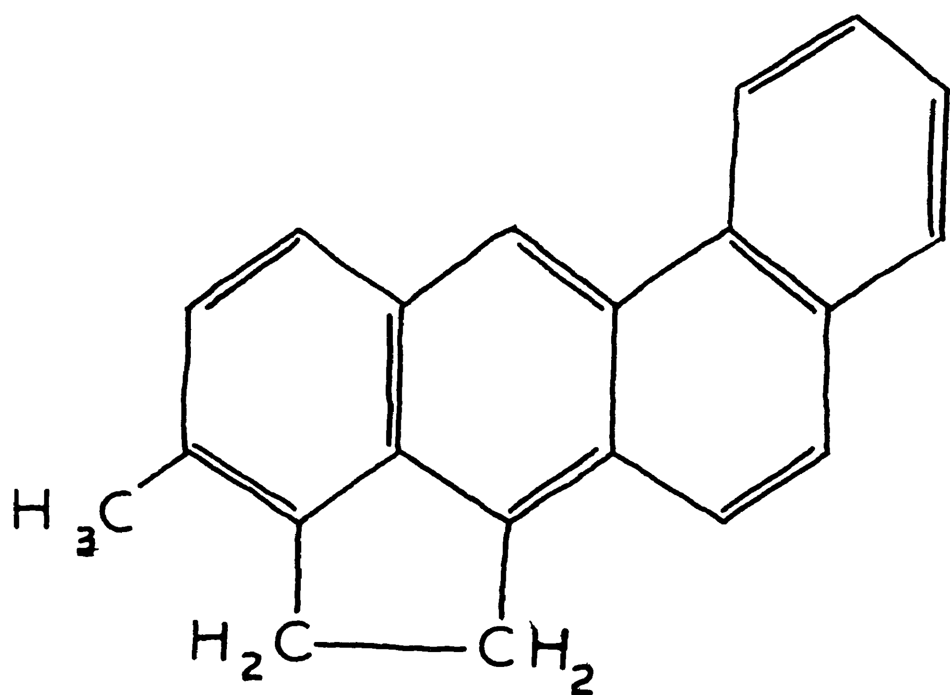


Fig. 2 20-METHYCHOLANTHRENE

itself was only very weakly active. The presence of one or two methyl groups, or an additional benzene nucleus, in the 1,2-benzanthracene molecule often resulted in highly potent carcinogens, while the presence of ethyl or propyl groups was less effective. The most favorable sites for the location of the substituent groups were at carbon atoms 9 and 10; less active hydrocarbons were obtained when carbon atoms 5 and 6 were substituted similarly (see Fig. 3).

The relationship between structure and carcinogenic activity can be seen by comparison of structural formulae (see Figs. 1, 2, and 3). The three most potent carcinogenic hydrocarbons are 20-methylcholanthrene, 3,4-benzpyrene, and 9,10-dimethyl-1,2-benzanthracene. Although the sterol numbering system for 20-methylcholanthrene and the pyrene system for 3,4-benzpyrene are different from the anthracene system, it is apparent from observation of chemical structure that the carcinogenic activity of these compounds results from substitution of aliphatic groups or aromatic rings at the effective positions in the 1,2-benzanthracene ring system.

The carcinogenic hydrocarbons are insoluble in water, but are soluble in lipids and organic solvents. They are applied, usually in dilute solution in lard or benzene, by painting the skin of the test animal (usually the mouse or rabbit), by subcutaneous injection (rat or mouse), or by intraperitoneal injection (rat or mouse) (23, 18, 2). The application by painting is repeated, if necessary, one or more times a week until the appearance of a tumor occurs, whereas with the injection procedure usually only one dose is necessary. The interval between primary application of the material and the appearance of the

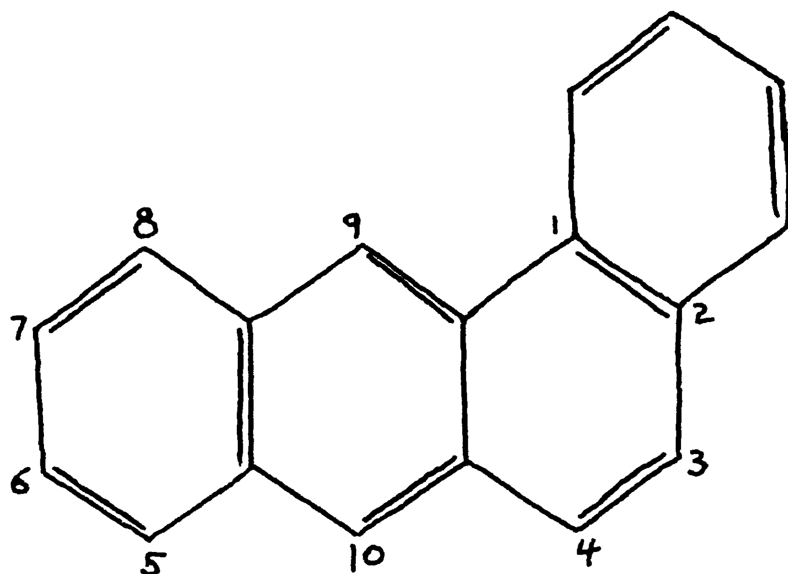


Fig. 3 1,2-BENZANTHRACENE

tumor is termed the latent period of the hydrocarbon. Skin painting of mice has shown that the most active hydrocarbon, 20-methylcholanthrene, has a latent period of about two and a half months or less, while the less active agents require nine months or longer to produce tumors under the same conditions (18). The potency of any particular hydrocarbon is considered to be a function of both the latent period and the percentage of animals in which tumor formation occurs; thus the most active hydrocarbons require less time for the development of tumors and the percentage of tumors produced is ninety to one hundred. The hydrocarbon-induced tumors occurred primarily at the site of application, although leukemia (48), and tumors of the lung (1) and liver (6) have been reported to be associated conditions. Metastases to other portions of the body have been reported by Cook (6) and Parsons (48); tumors could also be transplanted from one animal to another of the same species (51).

Whether any of the known carcinogenic hydrocarbons is involved in spontaneous cancer is not known. Cook and Haslewood (7) suggested that cholesterol or a bile acid may be transformed by abnormal metabolism into a cancer-producing hydrocarbon. This hypothesis is supported by the fact that such transformations can be carried out by relatively simple chemical reactions, such as the conversion of cholic acid to 20-methylcholanthrene in the laboratory. The similarity in chemical structure of this carcinogen to the steroids led to the suggestion by Fieser (18) that transformation of normal metabolic substances, such as corticosterone and oestrone, into carcinogenic compounds may occur

in the body. However, there has been no direct experimental proof that such transformations do occur under physiological conditions, although similar requisite reactions of side chain degradations and aromatization do occur in the animal body. To date, no carcinogenic hydrocarbons have been isolated from tumor tissue; the probable transient but effective existence of minute amounts (less than one milligram) of such a product at some time prior to tumor formation may well be the explanation. On the other hand, no available evidence excludes the possibility of such transformations as being the cause of some forms of cancer. A report was published by Druckrey, Richter, and Vierthaler (16) on the <u>in vitro</u> transformation of dehydrohorcholene, a compound related to cholic acid, to carcinogenic substances by cultures of <u>Escherichia coli</u> isolated from patients with rectal cancer. However, no confirmation of this work has been found. The injection of large amounts of oestrogenic hormones into mice does cause the formation of mammary tumors (22, 42). Loeb, (42), however, considered the oestrogenic substances to be distinct from the carcinogenic hydrocarbons, since the former as a rule are not carcinogenic, and the latter may or may not be oestrogenic.

That these hydrocarbons are involved in spontaneous cancer is only a possibility at this time; however, they definitely incite the production of cancer experimentally, as seen by the reports published since 1915. It becomes of interest, therefore, to develop a method of immunization against experimental hydrocarbon carcinogenesis, and furthermore, to determine if such an immunization could be made effective against either spontaneous or hormone-induced carcinogenesis.

The development of immunity in animals against hydrocarbons cannot be accomplished by injection of the hydrocarbons themselves, since, according to Zinsser, Enders, and Fothergill (56), only proteins, certain carbohydrates and carbohydrate-lipoid complexes are antigenic. An attempt to sensitize guinea pigs for anaphylactic shock with 1,2,5,6-dibenzanthracene was found to be unsuccessful by Landsteiner and Jacobs (37). Early in the study of immunology, it was discovered by Bordet (3) that native proteins are antigenic and therefore capable of inducing the production of serum antibodies. The method, introduced by Landsteiner and Lampl (36), of conjugating non-protein, non-antigenic compounds with antigenic proteins was a great step forward in the study of serological reactions. They were able to produce azoproteins by conjugation of benzene diazonium halides with horse and rabbit serum proteins. Antiserum produced in the rabbit against horse azoprotein reacted not only with the homologous antigen, but also with the rabbit azoprotein antigen. Similarly, mixing serum against rabbit azoprotein with the horse azoprotein also resulted in precipitation. In order to determine the specificity of these cross reactions, Landsteiner and Lampl made azoproteins from the para-amino derivatives of benzoic, sulfonic, and phenyl arsenic acid. Sera against the azoprotein of each acid precipitated strongly with azoproteins made from different proteins but from the same acid; the reactions of these sera with azoproteins made from the other acids were weak or negative. These aromatic acids conjugated with proteins differed from each other in the nature of the acidic group only, yet antiserum against benzoic acid horse

serum protein reacted strongly with benzoic acid rabbit serum protein, weakly with sulfonic acid rabbit serum protein, and not at all with phenyl arsenic acid rabbit serum protein. These investigators showed, therefore, that the extent of serological cross reactions among azoproteins was dependent on the similarity of the chemical groups attached to the proteins.

Further experimentation was carried out by Landsteiner and Lampl (39) and by Landsteiner (34) on the dependence of serological specificity on chemical structure. Amino groups were substituted in the aromatic acids mentioned above at the ortho, meta, and para positions; diazotization and conjugation with proteins then were conducted. The antisera to azoproteins of these substituted acids were specific, not only for the particular acid group, but also the particular spatial arrangement of the substituted antigen. For instance, antisera against the meta-aminobenzoic acid azoprotein reacted with proteins conjugated with the same compound, but not with proteins conjugated with para-aminobenzoic acid, meta-aminosulfonic acid, para-aminosulfonic acid, or para-aminophenyl arsenic acid. The presence of methyl, methoxyl, nitro, or bromine groups in the benzene ring did not give rise to such specific antigens, as cross-reactions with antisera to these compounds occurred, albeit only weakly.

These determinant groups introduced into the protein molecule have been termed "haptenes." A haptene is defined as a substance which is not capable of producing antibodies when injected alone, but which does react with antibodies produced by the injection of a conjugate of an antigenic protein with the haptene.

The experiments of Landsteiner showed the occurrence of a reaction between haptene and antisera by the precipitation of antisera in the presence of a specific haptene-protein conjugate. That the haptenes need not be combined with proteins to r eact with antisera later was shown by Landsteiner and his colleagues (35, 34, 40). These workers were able to show that although the haptene, or simple derivatives thereof, usually would not cause the precipitation of antisera, they would inhibit the formation of precipitates when the antisera were mixed with a suitable haptene-protein conjugate. The strength of the precipitation reaction between antisera against meta-aminobenzoic acid and the appropriate test antigen was markedly reduced by the addition of benzoic acid, or of derivatives of this compound, to the antigen-antibody mixture. The precipitation was completely inhibited by addition of meta-nitro, -brom, -chloro, and -methyl-benzoic acid, and partially inhibited by meta-amino and -hydroxy-benzoic acid, and by the benzoic acid itself. These haptenes showed a less pronounced chemical specificity than the azoproteins, but their use eliminated the possibility that the protein portion of the antigen influenced the specificity.

Since the coupling of the diazonium compounds with the proteins occurs with the tryosine and histidine side chains, tryosine was coupled with the amino-benzoic acid haptenes and the resulting compounds were tested as inhibitors. (34). Smaller quantities of these tyrosine-haptene compounds than of the haptenes themselves were required to inhibit the precipitation reactions.

Landsteiner and van der Scheer (41) found that conjugates of hydro-

carbons, such as butyl benzene and anthracene, with proteins acted only as weak antigens, showing slight specificity due to the determinant group, or haptene. It should be recalled that Landsteiner and Lampl had demonstrated (39) that the single benzene ring could act as a haptene, since diazotization of aniline and linkage to proteins served to give an effective antigen.

As mentioned above, Landsteiner's method of haptene linkage to protein was by the reaction of a diazonium compound with the tyrosine and histidine side chains of the protein. A different method of haptene linkage to proteins was used by Hopkins and Wormall (27, 28). The reaction of phenyl isocyanate with serum proteins or casein resulted in the conjugation of the two substances through a carbamido- (also called ureido) linkage, apparently involving only the free epsilon-amino groups of the protein. The phenyl groups thus attached to the proteins acted as determinant groups, as demonstrated by typical cross-reaction precipitation tests. By using phenyl-carbamido derivatives of lysine, glycine, and alanine as inhibitors of the precipitation reaction (29), they found that the reaction was inhibited completely by the lysine derivative, and incompletely by the other two derivatives. This supported their earlier view that phenyl-carbamido-lysine was the characteristic group of the phenyl-carbamido proteins.

Creech and Franks (12) used this carbamido linkage to prepare antigens composed of proteins and anthracene or 1,2,5,6-dibenzanthracene. Rabbit sera prepared against anthranyl-carbamido-casein formed precipitates in the presence of anthranyl-carbamido-globulin, and sera against the globulin derivative also reacted with the casein

derivative of anthracene. Antisera against 1,2,5,6-dibenzanthryl-carbamido-casein were precipitated not only with the homologous antigen, but also with 1,2,5,6-dibenzanthracene derivatives of globulin and albumin, and with the anthranyl-carbamido-globulin to a lesser degree. These results showed that the carbamido proteins had acquired a new specificity because of the haptene determinant groups. Injection of mice, rats, and rabbits with casein derivatives of the hydrocarbons did not result in carcinogenesis.

In a later paper, Franks and Creech (21) described a series of experiments on the immunization of mice with 1,2,5,6-dibenzanthranyl-carbamido-casein in an attempt to prevent tumors produced by 1,2,5,6-dibenzanthracene. One hundred control mice were injected with the 1,2,5,6-dibenzanthracene only; one hundred and fifty mice were injected with the 1,2,5,6-dibenzanthranyl-carbamido-casein over a seven week period which ended fourteen days prior to the injection of the carcinogenic hydrocarbon itself. A large number of the mice in both series died of intercurrent infection during the course of the experiment, thus reducing the statistical validity of the results. Apparently, because of the presence of traces of uncombined 1,2,5,6-dibenzanthranyl isocyanate (subsequently found unexpectedly to be carcinogenic) in the first preparation of hydrocarbon conjugate used for immunization, tumors developed in some cases at the site of the injection of the immunizing antigen. Despite these two difficulties, the results did show some evidence that injections of 1,2,5,6-dibenzanthranyl-carbamido-casein reduced susceptibility to carcinogenesis by the hydrocarbon, since thirty percent of the immunized mice and seventeen percent of the control mice not only survived over the mean time of the appearance of tumor in the controls, but were also found, on death or sacrifice, to have no tumors.

EXPERIMENTAL

PART I

I. PURPOSE

The purpose of the first part of the experimental studies was to determine the comparative haptene activity of three hydrocarbons, namely, 1,2-benzanthracene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene. All three hydrocarbons previously had been shown to be capable of haptene activity* but no extensive immunological studies had been conducted. One aim of the experiments was the development of antisera which would react not only with the hydrocarbon used in immunization, but which also would show cross reactions with other similar hydrocarbons. Such moderately non-specific antibody reactivity would be desirable, if not necessary, in any immunization against spontaneous tumors assuming that the inciting agents may be hydrocarbons of unknown, though possibly similar, chemical structure.

Immunization against the hydrocarbons required conjugation of these compounds with protein, since the hydrocarbons are not antigenic when injected alone. Haptene activity of a hydrocarbon would be demonstrated if antisera against a hydrocarbon-protein conjugate reacted with a conjugate of the same hydrocarbon with a different protein. The strength of this reaction would be a measure of the ability of the hydrocarbon to act as a haptene group. A low degree of specificity of the haptene would be indicated if these antisera would combine also with protein

* Unpublished preliminary work by Creech, Cheever and Coons at Harvard University.

conjugates prepared from hydrocarbons of similar structure. Investigations were conducted to evaluate the three hydrocarbons in terms of their haptene activity.

II. ANTIGENS

The antigens used for immunization were synthesized by Dr. Hugh J. Creech, formerly of the Chemistry Department of the University of Maryland. The methods used for their preparation and analysis are described by Creech and Jones (11, 13, 14, 15, 30). The three main hydrocarbons used for the formation of antigenic protein conjugates were 1,2-benzanthracene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene. These hydrocarbons were nitrated and the mono nitro compounds were reduced to the amines. The addition of phosgene to a benzene solution of the amines resulted in the formation of crystalline isocyanates of the hydrocarbons. The position of the isocyanate group (-N=C=O) on the hydrocarbon varied with the individual compound. The isocyanate group was at carbon atom 10 with 1,2-benzanthracene, at carbon atom 5 with 3,4-benzpyrene, and at carbon atom 9 with 1,2,5,6-dibenzanthracene (see Figs. 4, 5, and 6). Other isocyanates were prepared by special methods.

The isocyanates were conjugated with horse serum albumin which had previously been separated from horse serum according to the procedure of McMeekin (44). The linkage of hydrocarbon to albumin was carried out by addition of a dioxane solution of hydrocarbon-isocyanate to a cold aqueous-dioxane solution of the protein buffered at pH 8.2 - 8.3. The reaction occurred between the isocyanate group of the hydrocarbon and the free epsilon-amino groups of the protein,

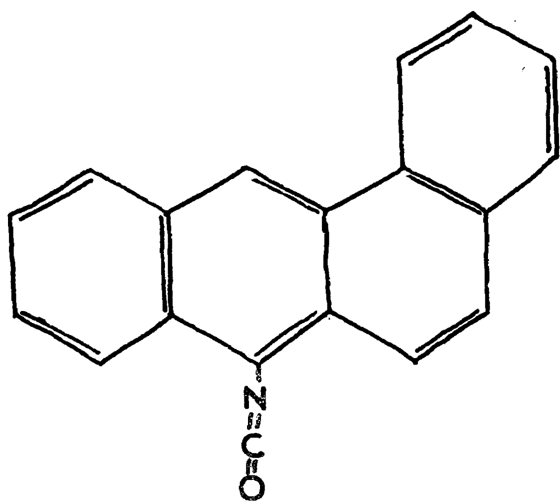


Fig. 4 1,2-BENZANTHRYL-10-ISOCYANATE

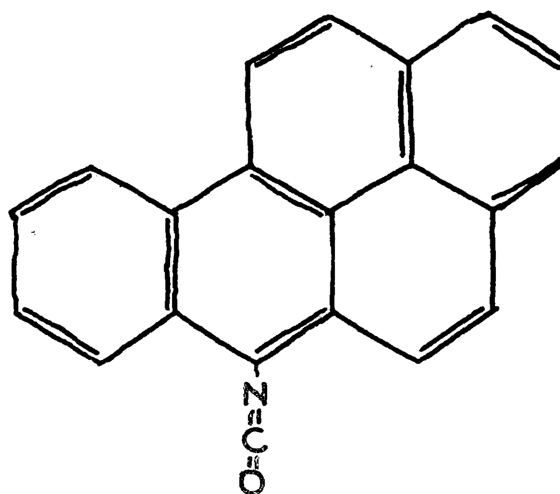


Fig. 5 3,4-BENZPYRENYL-5-ISOCYANATE

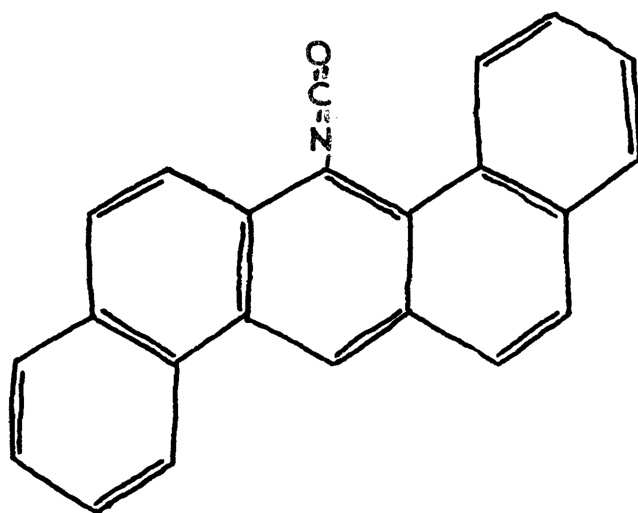
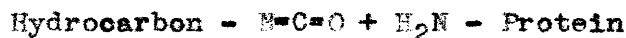
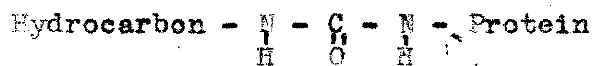


Fig. 6 1,2,5,6-DIBENZANTHRYL-9-ISOCYANATE

resulting in a carbamido linkage:





carbamido
linkage

The conjugates were purified by dialysis, centrifugation, filtration, and precipitation with ammonium sulfate and acetone.

Spectrophotometric analyses, based on the absorption of ultraviolet light by the hydrocarbon-protein conjugates, were used to determine the number of hydrocarbon groups attached to each molecule of horse serum albumin. These analyses were carried out by Dr. R. Norman Jones of Harvard University and Queens University, Kingston, Ontario. The results for the conjugates used as antigens are as follows:

3,4-benzpyrenyl-5-carbamido-horse serum albumin--19 groups
(test antigen)
3,4-benzpyrenyl-5-carbamido-horse serum albumin-- 4 groups
(injection antigen)

1,2-benzanthryl-10-carbamido-horse serum albumin--38 groups
(test and injection antigen)

1,2,5,6-dibenzanthryl-9-carbamido-horse serum albumin--
21 groups (test and injection antigen).

The hydrocarbon-protein conjugates were dissolved in physiological saline solution to give a concentration of ten mg. per cc. Unconjugated horse serum albumin in the same concentration was used as the fourth antigen, so that control sera would be available for the serological investigations. All of the antigen solutions were stored with preservative in sterile rubber-capped bottles in the refrigerator during the entire course of the experiments; no bacterial contamination was noted at any time.

III. IMMUNIZATION PROCEDURE Series I

A. Animals

Eleven albino rabbits were obtained from a dealer in laboratory stock. Each rabbit weighed approximately three pounds at the beginning of the experimental work. Seven were male, and four were female. Their diet consisted of Purina rabbit chow, supplemented by cabbage, lettuce, and carrots.

The animals were divided into five groups, on the basis of the antigen used for injection.

TABLE I

ANTIGEN GROUPS OF RABBITS

Group No.	No. of rabbits	Antigen
I	2	Horse serum albumin
II	2	3,4-Benzpyrenyl-5-carbamido-horse serum albumin
III	3	1,2-Benzanthryl-10-carbamido-horse serum albumin
IV	3	1,2,5,6-Dibenzanthryl-9-carbamido-horse serum albumin
V	1	None

B. Needles and syringes

Becton-Dickinson or Acme stainless-steel needles were employed for all injections and bleeding. Each needle was placed in a test tube containing, in the butt, absorbent cotton in which the tip of the needle rested; the tubes were plugged and sterilized in the autoclave for fifteen to twenty minutes at fifteen pounds pressure, and 121° C. Gauge no. 24 needles were used for intravenous injections, and Gauge no. 22 and 20 needles for the intraperitoneal injections.

The syringes were of the Luer type; they were individually wrapped in gauze and brown paper, and sterilized in the autoclave at the same time and temperature as the needles. Two-cc. syringes were used for the small injections, and five-cc. syringes for the large injections.

After being used for injection, the syringes and needles were washed thoroughly in physiological saline and in running tap water before being sterilized. After autoclaving, they were allowed to dry for several hours before being used again.

C. Injection series

The antigen solutions were allowed to reach room temperature before injection, so that large volumes of cold solution would not be injected. The outer marginal ear vein was used for intravenous injections. The hair over the vein was carefully removed with a razor blade, and the site swabbed with ninety-five percent alcohol. A two or a five-cc. syringe and a gauge no. 24 needle were employed. The needle was inserted in the vein, and the antigen solution injected slowly. The rate of injection was approximately one cc. in twenty to thirty seconds.

For intraperitoneal injections, a site was prepared by shaving an area of about two square centimeters on the abdomen of the rabbit. Tincture of green soap was used to facilitate removal of the hair. After shaving, the site was swabbed with ninety-five percent alcohol. A five-cc. syringe and a gauge no. 22 or 20 needle were employed. The antigen was injected into the peritoneal cavity at a more rapid rate than during intravenous injection, since there is no resistance to the flow of the liquid into the cavity.

Each rabbit was injected with the appropriate antigen three times per week for five weeks. The protocol for the injection series is shown in Table II.

TABLE II

RABBIT INJECTION SERIES

Week no.	No. of injections per week	Mode of injection	No. of cc. sol'n injected	Mgs. antigen injected	Mgs. antigen per week
1	3	I.V.	1	10	30
2	3	I.V.	2	20	60
3	3	I.V.	3	30	90
4	3	I.P.	4	40	120
5	3	I.P.	5	60	150
		Total mgs. antigen injected:			450

None of the rabbits showed ill effects after the injections. There was an average weight gain of two to three pounds during the eight-week period from receipt of the animals until the final bleeding. No tumors were found at the injection sites or elsewhere in the animals.

D. Preliminary bleeding and titration

One rabbit from each group was selected for preliminary titration of antibodies. Four days after the last injection, the ear vein of each rabbit was cut, and approximately five cc. of blood was collected in a sterile fifteen-cc. centrifuge tube. The blood was allowed to clot at room temperature for one hour, and then was placed in the refrigerator overnight. After centrifugation, the supernatant serum was removed with a pipette.

Precipitation tests were run to determine the antibody activity of these sera. The antisera were diluted to twice their volume with physiological saline solution (0.85% sodium chloride in distilled water). The antigen solutions, containing ten mg. antigen per cc., were mixed with saline solution to give serial dilutions of 1-10, 1-50, 1-100, 1-200, 1-400, 1-800, 1-1000 and thereafter by increments of 1-200 up to a final dilution of 1-3000. In tubes of 10 mm. by 75 mm., one-tenth cc. of antibody was mixed with two-tenths cc. of each dilution of the same antigen used for immunization of the rabbit from which the serum was taken. The tubes were shaken thoroughly, and placed in the 46°C. incubator for one hour and fifteen minutes. Precipitation occurred only in the tubes containing horse serum albumin and rabbit serum prepared against that antigen. Therefore, the tubes were placed overnight in the refrigerator. After refrigeration, the presence of precipitate in the tubes was observed visually using shaded incandescent light. In order to simplify the composition of this and other tables, the names of the antigens will hereafter be abbreviated, in both text and tables, as follows:

HSA - horse serum albumin

BP-HSA - 3,4-benzpyrenyl-5-carbamido-horse serum albumin

10-HSA - 1,2-benzanthryl-10-carbamido-horse serum albumin

DBA-HSA - 1,2,5,6-dibenzanthryl-9-carbamido-horse serum albumin

The results of the preliminary titrations are shown in Table III.

TABLE III

PRELIMINARY TITRATIONS OF ANTISERA

Antiserum against	Highest dilution of antigen showing precipitation
HSA	3000 *
BP-HSA	200
10-HSA	1400
DBA-HSA	400

* Highest dilution tested

Antibodies demonstrated by such precipitation tests are called precipitins; those demonstrated by other serological procedures are also named according to the method used, e.g. lysins, agglutinins. Since some information, albeit it of questionable significance, may be obtained by comparison of the greatest dilutions of antigen producing a slight precipitate with a constant amount of antiserum, these figures have been recorded above and in certain subsequent tables for the sake of completeness only.

Six rabbits were selected for further injections; they included the four which had been tested serologically, and two others which had not been bled. These two rabbits included one member each of the 10-HSA and DBA-HSA groups. The six rabbits each received an additional course of three intraperitoneal injections, which began seven days after the regular course. These injections were spaced at three-day intervals; the first was two cc. (twenty mg.); the second, two cc. (twenty mg.); and the third, five cc. (fifty mg.). The total amount of antigen injected into each of these six rabbits therefore increased from four hundred and fifty mgs. to five hundred and forty mgs. The

four remaining immunized rabbits, which included one member of each antigen group, were not reinjected, but were bled out according to the procedure described below.

E. Final bleeding

The rabbits were bled from the heart ten to thirteen days after the last injection of antigen. A fifty-cc. syringe and a gauge no. 18 needle were prepared and sterilized by the methods described in section III. The skin over the thorax was shaved, and swabbed with tincture of iodine. The region of maximum pulsation was determined. The needle was inserted through the chest wall into the heart, and blood was pulled into the syringe by gentle suction.

The blood in the syringe was run into sterile Erlenmeyer flasks, and allowed to clot at room temperature for an hour. It was then placed in the refrigerator overnight. The supernatant serum was pipetted off the clot, and centrifuged to produce clear serum. Sterile rubber-capped bottles were used for refrigerator storage of the serum; two or three drops of a 1:1000 dilution of merthiolate were added as a preservative.

Three of the rabbits were bled twice. The first bleeding gave approximately forty cc. of blood; the second bleeding, which resulted in death of the animal, gave from seventy-five to ninety cc. The other eight rabbits were bled only once; the amount of blood removed varied from seventy-five to ninety-five cc. This loss of blood caused death of the animals. Table IV shows the designations of the sera, and the number of days after injection that each serum was obtained.

TABLE IV

DESIGNATION OF IMMUNE SERA

Antigen	Rabbit tag no.	Designation of serum	Days after last injection
HSA	1	HSA-1a	10
"	1	HSA-1b	16
"	2	HSA-2a	10
"	2	HSA-2b	16
BP-HSA	3	BP-HSA-1a	12
"	4	BP-HSA-2a	10
"	4	BP-HSA-2b	15
10-HSA	5	10-HSA-1a	10
"	6	10-HSA-2a	13
"	7	10-HSA-3a	13
DBA-HSA	8	DBA-HSA-1a	12
"	9	DBA-HSA-2a	11
"	10	DBA-HSA-3a	10
None	11	Normal	

F. Autopsy

Autopsies were performed on one rabbit of each group which received a hydrocarbon-protein antigen. Illumination of the opened carcass with ultra-violet light showed colored fluorescence of the spleen and several patches of lymphatic tissue of the omentum. The hydrocarbon-protein conjugates had previously been shown by Creech and Jones (15) to be highly fluorescent. The localization of the injected antigens in the lymphatic tissue was not surprising, since it is this tissue which is considered to be primarily important in the removal of foreign protein from the blood, and possibly in the formation of antibodies as well.

No tumors were seen under gross macroscopic observation of the organs of the thorax and abdomen.

III. SEROLOGICAL STUDIES

A. Homologous antigens

The activity and specificity of the antisera were determined by precipitation tests. The required amount of each antiserum was diluted with an equal volume of saline solution. One-tenth cc. of the diluted serum was pipetted into tubes measuring 10 mm. by 75 mm. The antigen which had been used for immunization (homologous antigen) was diluted with saline solution as follows: 1-10, 1-50, 1-100, 1-200, 1-400, 1-800, 1-1000 and thereafter by increments of 1-200 up to a dilution of 1-3000, or higher if necessary. It should be pointed out that the immunizing antigens were used in a concentration of 10 mgs. per cc., or a 1-100 concentration. Therefore, a dilution of 1-3000 of antigen solution actually represented a dilution of 1-300,000 of the antigen itself. Two-tenths cc. of each dilution of antigen were added to the one-tenth cc. of diluted serum. The mixtures were incubated in a 46°C. incubator for one hour. However, several duplicate tubes incubated under different conditions, i.e. room temperature, 37°C. incubator, or 37°C. water bath, showed no significant differences in the precipitation reactions. After incubation, the tubes were placed in the refrigerator overnight, and were read the following day by shaded incandescent light. The amount of precipitate was recorded as: very faint trace (vftr), faint trace (ftr), trace (tr), ±, +, +±, ++, ++±, +++, +++±, and ++++. The long gradation of readings was used in order to determine as accurately as possible the dilution of antigen at which maximum precipitation occurred.

The results of these experiments are shown in Tables V, VI, VII, and VIII. Table V shows the reactions between horse serum albumin and

the rabbit sera prepared against that antigen. It should be recalled that serum HSA-1a and serum HSA-1b were taken from the same rabbit, but that the HSA-1b serum was removed six days after the first. The same sequence held for the HSA-2a and HSA-2b sera. Comparison of the results for the a and b sera for each rabbit shows that there was but little difference in the readings. Such small variations might have been due to experimental errors in pipetting or in reading the amount of precipitate. Apparently, the second bleeding serum which was not markedly different in antibody content from the first serum, obtained only ten days after the last antigen injection.

TABLE V

AMOUNT OF PRECIPITATE USING HSA ANTISERA AND HOMOLOGOUS ANTIGEN

Antigen dil. (0.2 cc.)	Antiserum (0.1 cc.) HSA-1a	HSA-1b	HSA-2a	HSA-2b
1- 10	++	++	++	++
50	++	++	+++	++
100	++	++	++++	+++
200	++	+++	++++	++++
400	+++	+++	++++	++++
800	+++	+++	+++	+++
1000	++	++	++±	++±
1200	++	++	++±	++
1400	++	++	++	++
1600	++	++	++	++
1800	+±	+±	++	+±
2000	±	±	++	+±
2200	±	±	+±	+±
2400	±	tr	+±	+
2600	tr	tr	+	+
2800	tr	tr	+	+
3000	tr	tr	±	±
3200	tr	tr	±	±
3400	tr	tr	tr	tr
3600	tr	ftr	tr	tr
3800	ftr	ftr	tr	tr
4000	-	-	tr	ftr
4200	-	-	ftr	ftr
4400	-	-	ftr	ftr
4600	-	-	vftr	vftr
4800	-	-	-	-

At the lowest dilutions of horse serum albumin (i.e. 1-10, 1-50), the yield of precipitate was less than at the slightly greater dilutions of antigen (i.e. 1-400, 1-800), where it reached a maximum. Thereafter, as the amount of antigen was decreased by further dilution, the amount of precipitate also decreased; at very high dilutions of antigen, no precipitation occurred at all. Three zones of antigen-antibody ratios were therefore in evidence: (1) zone of antigen excess, (2) zone of neutrality, or optimum proportions, and (3) zone of antibody excess. This zone phenomenon is characteristic in precipitation reaction in general (4). Comparison of the sera of the two rabbits showed that the optimum amount of antigen, yielding greatest precipitation, was different with the two sera. For 0.05 cc. of serum HSA-1 (results of 1a and 1b combined) it was 0.2 cc. of a 1-400 dilution of the antigen solution, whereas with the same quantity of serum HSA-2 (results of 2a and 2b combined), the optimum amount of antigen was 0.2 cc. of a 1-200 dilution. Since the latter serum combined with more antigen and formed heavier precipitates at its optimum ratio than did the former serum, the content of precipitating antibodies of sera HSA-2a and HSA-2b appeared to be greater than that of sera HSA-1a and HSA-1b.

The highest dilution of antigen which still gave slight precipitates on reaction with antisera HSA-1a and -1b was 1-3800, and with antisera HSA-2a and -2b it was the 1-4600 dilution of antigen.

The precipitation reactions of the rabbit sera prepared against BP-HSA when mixed with the homologous antigen are shown in Table VI. The sera from the two rabbits differed rather markedly in antibody content. The BP-HSA-1a serum formed heavier precipitates than the BP-HSA-2a or 2b sera.

TABLE VI

AMOUNT OF PRECIPITATE USING BP-HSA ANTISERA AND HOMOLOGOUS ANTIGEN

Antigen dil. (0.2 cc.)	Antiserum (0.1 cc.) BP-HSA-1a	BP-HSA-2a	BP-HSA-2b
1- 10	++	+±	++
50	++	±	±
100	+±	±	±
200	+++	tr	tr
400	++	tr	tr
800	++	tr	ftr
1000	++	ftr	-
1200	+±	-	-
1400	±	-	-
1600	∓	-	-
1800	∓	-	-
2000	±	-	-
2200	±	-	-
2400	tr	-	-
2600	tr	-	-
2800	ftr	-	-
3000	ftr	-	-

The end titer of BP-HSA-1a was 1-3000; those of the BP-HSA-2a and -2b sera were titer 1-800 and 1-1000. As with the HSA antisera, the antisera from the second bleeding appeared to be only very slightly different from the first serum obtained from the rabbit.

The most striking fact about the results in Table VI, however, is the sharp drop in antiserum potency, as compared with the results in Table V. The conjugation with hydrocarbon apparently greatly reduced the antigenic properties of the serum albumin, even though only four 3,4-benzpyrenyl groups were introduced into each protein molecule in the conjugate used for immunization.

Antisera prepared by immunization with 10-HSA were tested against the homologous antigen. The results are given in Table VII. These three antisera were obtained from different rabbits. Individual differences in the sera were evident by the variation in the amount of the

TABLE VII

AMOUNT OF PRECIPITATE USING 10-HSA ANTISERA AND HOMOLOGOUS ANTIGEN

Antigen dil. (0.2 cc.)	Antiserum (0.1 cc.) 10-HSA-1a	10-HSA-2a	10-HSA-3a
1- 10	+	tr	tr
50	±	++	+±
100	+±	++	++
200	+±	++±	++±
400	+±	+++	+++
800	+±	++±	+++
1000	+∓	++	+++
1200	+∓	+	++-
1400	+	+	+
1600	±	±	±
1800	±	tr	tr
2000	±	tr	tr
2200	tr	tr	tr
2400	tr	tr	ftr
2600	tr	tr	ftr
2800	tr	ftr	ftr
3000	tr	ftr	ftr
3200	tr	ftr	ftr
3400	ftr	ftr	ftr
3600	ftr	ftr	-
3800	ftr	ftr	-
4000	ftr	-	-
4200	ftr	-	-
4400	ftr	-	-
4600	-	-	-
4800	-	-	-

maximum precipitate. A good part of the antigenic potency of the protein was retained after conjugation, although thirty-eight 1,2-benzanthryl groups were introduced into the horse serum albumin molecule. Two of the 10-HSA antisera which showed heavy precipitates in the zone of optimum proportions, were only slightly less potent than the antisera against the unaltered protein when tested with the homologous antigens.

Table VIII shows the precipitation reactions of the three antisera

against DBA-HSA. Only very slight precipitation occurred in any of the tubes. These sera did not compare favorably in potency with the antisera against the other two hydrocarbon-protein conjugates. Twenty-one 1,2,5,6-dibenzanthranyl groups had been linked to the protein; apparently the conjugation with the hydrocarbon caused a considerable modification of the antigenicity of the albumin.

TABLE VIII

AMOUNT OF PRECIPITATE USING DBA-HSA ANTISERA AND HOMOLOGOUS ANTIGEN

Antigen dil. (0.2 cc.)	Antiserum (0.1 cc.)		
	DBA-HSA-1a	DBA-HSA-2a	DBA-HSA-3a
1- 10	ftr	ftr	ftr
50	ftr	tr	ftr
100	ftr	±	tr
200	ftr	tr	ftr
400	tr	tr	ftr
800	ftr	ftr	ftr
1000	-	ftr	-
1200	-	ftr	-
1400	-	vftr	-
1600	-	vftr	-
1800	-	vftr	-
2000	-	vftr	-
2200	-	vftr	-
2400	-	-	-
2600	-	-	-
2800	-	-	-
3000	-	-	-

B. Horse serum albumin

All nine sera which had been prepared by immunization with hydrocarbon-horse serum albumin conjugates were tested against horse serum albumin. This was done to determine how great a modification of the protein antigenicity had occurred in conjugation with hydrocarbons. It was expected, in accordance with the results of Tables V and VIII,

that the antisera against the conjugates would not yield as heavy precipitates with the albumin as did the sera against the horse serum albumin itself.

The antisera were diluted with equal volumes of saline solution, and one-tenth cc. of each diluted serum was mixed with two-tenths cc. of each of the twenty-six dilutions of horse serum albumin. These antigen dilutions were made according to the progression described above, i.e. from 1-10 to 1-4800. Control tubes were set up with antiserum and saline solution, antigen and saline solution, and normal serum and saline solution. No precipitation occurred in any of the control tubes. The results of these experiments are shown in Table IX. Figures for the sera against horse serum albumin are included for comparison.

According to the figures in Table IX, none of the antisera against the conjugated albumins yielded as much precipitate as did the antisera against the unconjugated protein. The 10-HSA serum group appeared to have more precipitins against the albumin than did either of the other two serum groups. This indicated less modification of the antigenic structure of the albumin on conjugation with 1,2-benzanthracene than with 3,4-benzpyrene or 1,2,5,6-dibenzanthracene. This is in agreement with the results against the homologous antigens shown in Tables V to VIII.

A lack of agreement between the serological methods, i.e. end-titer method and optimum zone method, is indicated by the above figures. Antisera BP-HSA-1a and DBA-HSA-2a both showed +± readings at the optimum zone, yet the end titer of the former serum was 1-400 and of the

TABLE IX

PRECIPITATION REACTIONS USING HSA AS TEST ANTIGEN

Antiserum	Antigen dil. showing max. ppt.	Reading of maximum ppt.	Highest antigen dil. showing ppt.
HSA-1a	1-400, 800	+++	1-3800
-2a	100, 200, 400	++++	4600
BP-HSA-1a	10	+±	400
2a	10, 50	tr	100
2b	10, 50	tr	100
10-HSA-1a	200, 400	++±	4400
2a	2400	±	4400
3a	800	+	4800 *
DBA-HSA-1a	1000	+	3800
2a	1000	+±	4000
3a	200, 400	tr	1200

* Highest dilution tested

latter 1-4000. Conversely, two 10-HSA sera, 1a and 2a, had the same end-titer but markedly different precipitate readings at the optimum zone.

C. Heterologous Hydrocarbon conjugates

A third series of experiments was carried out to determine the extent of the reactions between these antisera and hydrocarbon-albumin conjugates not used for production of the antiserum under test. In the first group of experiments, one member of each group of antisera group was tested against the other antigens used for immunization, i.e. the conjugates of horse serum albumin. These tests were performed with the intention of confirming the results obtained by the first two series of experiments on the decrease of antigenicity of the albumin fraction of the conjugated protein.

In the second group, each antiserum was tested against bovine serum albumin and hydrocarbon conjugates of this protein. These were the first experiments aimed at determining the haptene group activity of the hydrocarbons.

1. Hydrocarbon - horse serum albumins as test antigens

Four antisera only were used in these experiments. One antiserum of each immunized group was used, namely, HSA-la, BP-HSA-la, 10-HSA-la, and DBA-HSA-la. The sera were diluted with saline solution to twice their volume, and one-tenth cc. of the diluted serum was mixed with two-tenths cc. of each dilution of antigen. The three conjugated antigens used were BP-HSA, 10-HSA, and DBA-HSA. Dilutions were made as previously described; the highest dilution of BP-HSA used was 1-3000, whereas the highest dilutions of both 10-HSA and DBA-HSA antigens were 1-4800. The antiserum and antigen in each tube were mixed, incubated, and placed in the refrigerator overnight.

Each antigen was tested against the three heterologous antisera; for example, BP-HSA antigen was mixed with antisera against HSA, 10-HSA, and DBA-HSA. The results of the reactions with the homologous antisera, i.e. BP-HSA-la, 2a, and 2b antisera, have been shown previously in Table VI. The same protocol was used for the other two conjugated antigens. The results of these experiments, shown in Table X, were practically opposite to those obtained in the other tests and were more in agreement with the expected results. The experiments previously described had indicated that loss of antigenicity of the horse serum albumin was greater in the BP- and DBA-conjugates than in the 10- conjugates, as measured by precipitation with both

TABLE X

PRECIPITATION REACTIONS USING HETEROLOGOUS HORSE SERUM ALBUMIN CONJUGATES AS TEST ANTIGENS

Test antigen	Antiserum	Antigen dil. showing max. ppt.	Reading of maximum ppt.	Highest dil. showing ppt.
BP-HSA	HSA-1a	1-10	++±	1-3000 *
"	10-HSA-1a	200	++±	2800
"	DBA-HSA-1a	1800	+	2800
10-HSA	HSA-1a	10, 50	tr	800
"	BP-HSA-1a	10	+	1400
"	DBA-HSA-1a	800	tr	3000
DBA-HSA	HSA-1a	200, 400	±	2000
"	BP-HSA-1a	10, 50	ftr	200
	10-HSA-1a	200, 400	+±	3200

* Highest dilution tested

homologous antigen and horse serum albumin. Analysis of the results in Table X indicates, however, that BP-HSA antigen was strongly precipitated by the HSA serum, while 10-HSA antigen yielded only traces of precipitate with this serum. In the interpretation of these seemingly conflicting results, consideration must be given to the fact that the antigens are being used in different ways. In the first case, comparison is being made among the antibodies produced by the injection of the antigens whereas in the second case, comparison is being made among the test antigens. The difference in activity of the albumin portion of the conjugates when used as injection and as test antigens does not appear to be due solely to the number of hydrocarbon groups introduced. The reasons for these differences will be advanced later in the thesis.

2. Hydrocarbon-bovine serum albumins as test antigens

As shown by the references cited in the section on the historical background of the thesis, haptene activity can be determined by using two different proteins, each containing either the same or a similar haptene. One such conjugated protein was used for immunization. Serum thus produced was tested against the other conjugated protein. When precipitation occurred, it was considered to be due to the antigenic specificity of the haptene alone, since the two proteins were different and did not give such cross reactions.

All of the antisera were first tested against dilutions of bovine serum albumin, in order to exclude the possibility of cross reactions between the two albumins. Precipitation did not occur with these sera, with the exception of one very faint trace reaction between a concentrated bovine serum albumin solution and an antiserum against horse serum albumin. Tests showing precipitation with a hydrocarbon-bovine serum albumin conjugate would be due entirely to the hydrocarbon portion of the conjugate.

The bovine serum albumin conjugates were synthesized by Dr. Creech, according to the method described in section II. Three such conjugates were prepared, namely, 3,4-benzpyrenyl-5-carbamido-bovine serum albumin (BP-Bov) containing nineteen hydrocarbon groups per protein molecule; 1,2-benzanthryl-10-carbamido-bovine serum albumin (10-Bov) with thirty-one groups; and 1,2,5,6-dibenzanthryl-9-carbamido-bovine serum albumin (DBA-Bov) with twenty groups. The antigens were used in saline solution, in a concentration of five mgs. per cc.

The first set of experiments to determine haptene activity of the hydrocarbons was carried out by using BP-Bov as the test antigen.

More antigen and antiserum were used for these reactions than for those previously described since it was expected that the precipitation would be less. Increasing the concentration of the reactants would increase the possibility of detecting precipitation. The antigen was diluted with saline solution, using a series of 1-2, 1-4, 1-8, 1-16, and so on up to 1-4096. To two-tenths cc. of each antigen dilution was added two-tenths cc. of antiserum diluted 1-2. The tubes were incubated, refrigeratred, and read by the previously described methods.

According to the results shown in Table XI, all of the immune sera against the hydrocarbon-horse serum albumin conjugates reacted with the BP-Bov antigen. Since it had been shown previously that these sera did not yield precipitates with bovine serum albumin alone, the precipitation with BP-Bov must have been due to combination with the 3,4-benzpyrene groups.

It is apparent from a comparison of the figures that the antisera against 10-HSA precipitated BP-Bov the most strongly, even more than the antisera against BP-HSA. This indicates that the serological activity of the hydrocarbon haptenes is not strictly specific, i.e. serum against one hydrocarbon conjugate would precipitate with conjugates of other hydrocarbons closely related in chemical structure. This was confirmed by the results in Table XII, where 10-Bov was used as the test antigen. The same procedures were used for these tests as for the BP-Bov reactions.

The 1,2-benzanthryl (denoted by "10") groups introduced appeared to show strong haptene group activity. The antisera produced by immunization with 10-HSA reacted strongly with the 10-Bov conjugate

TABLE XI

PRECIPITATION REACTIONS USING BP-BOV AS TEST ANTIGEN FOR HAPTENE ACTIVITY

Antiserum	Antigen dil. showing max. ppt.	Reading of maximum ppt.	Highest dil. showing ppt.
Normal	-	-	-
HSA-1b	-	-	-
BP-HSA-1a	1-1 *	tr	1-16
BP-HSA-2a	1 *	±	16
BP-HSA-2b	1 *	±	16
10-HSA-1a	2	++	512
10-HSA-2a	2, 4	±	256
10-HSA-3a	16, 32	+	128
DBA-HSA-1a	1, 2, 4	tr	16
DBA-HSA-2a	4	±	32
DBA-HSA-3a	4	±	16

* -undiluted antigen

TABLE XII

PRECIPITATION REACTIONS USING 10-BOV AS TEST ANTIGEN FOR HAPTENE ACTIVITY

Antiserum	Antigen dil. showing max. ppt.	Reading of maximum ppt.	Highest dil. showing ppt.
Normal	-	-	-
HSA-1b	-	-	-
BP-HSA-1a	1-8, 16	tr	64
BP-HSA-2a	4, 8	tr	16
BP-HSA-2b	4, 8, 16	tr	32
10-HSA-1a	64	++±	4096 *
10-HSA-2a	32	+	512
10-HSA-3a	64	+	4096 *
DBA-HSA-1a	16	tr	16
DBA-HSA-2a	8, 16, 32	tr	256
DBA-HSA-3a	8, 16 ≠	ftr	16

* -highest dilution tested
≠ -only dilutions showing precipitates

even in high dilution; this precipitation could have been due only to antibodies directed against the hydrocarbon group, since no reactions occurred between these 10-HSA antisera and bovine serum albumin alone, or between antiserum against horse serum albumin and the 10-Bov conjugate. The 3,4-benzpyrene and 1,2,5,6-dibenzanthracene showed weaker activity as haptenes, as shown by the smaller amount of precipitate with antisera against the BP-HSA and DBA-HSA conjugates.

Experiments then were conducted using 1,2,5,6-dibenzanthryl-9-carbamido-bovine serum albumin (DBA-Bov) as the test antigen for haptene activity. The same procedures were used, except that two-tenths cc. of undiluted, instead of diluted, serum were used in each tube. Heavy precipitation occurred with all sera, including the normal serum and that prepared against horse serum albumin. The precipitation was heaviest in the tube containing the most antigen, i.e. two-tenths cc. of undiluted antigen, and decreased with the lowering of antigen concentration. There was only occasional precipitation in any of the series beyond the 1-8 dilution of antigen. These tests were repeated several times, using only the four lowest dilutions of the DBA-Bov, with the same results.

A new solution of the same antigen preparation was made and tested. The results with the new solution were essentially similar. Occasionally, the tube containing the undiluted antigen was extremely turbid, but contained no precipitate, whereas the tube with the 1-2 dilution of antigen contained a considerable amount of precipitate. However, after shaking both tubes, the turbidity of the second tube was less than that in the first.

The amount of antiserum used was then halved to one-tenth cc. of undiluted serum, in an attempt to eliminate the non-specific precipitation reactions of the control sera. However, this procedure was of little value, since the normal serum and the serum against horse serum albumin still showed precipitates. The amount of precipitate was somewhat less than that with the sera against the conjugated antigens.

Synthesis of a new DBA-Bov preparation resulted in a compound forming slightly less opalescent solutions than the one which had been previously used. It did not yield quite as heavy precipitates, but the reaction was still not specific for haptene activity, since the control sera formed precipitates with the new preparation as well as with the old. A probable explanation is the greater insolubility of the DBA-Bov compared with the other conjugates.

D. Additional immunization series

The serological tests already performed had indicated that 10-HSA was the most satisfactory immunizing antigen, producing sera which reacted strongly with both protein and hydrocarbon moities of the conjugates. Therefore, two additional rabbits were immunized with this antigen. This was done to confirm the results, and to provide sufficient serum for the serological investigations to follow.

The procedure for the injections was the same as that described in section III. However, one rabbit received only three hundred and fifty mgs. of antigen; the last two intraperitoneal injections of fifty mgs. each were omitted. This was done to determine whether decreasing the amount of antigen would appreciably lower the antibody content of the serum. The rabbit was bled on three successive days,

starting with the ninth day after injection. The first two bleedings obtained approximately forty cc. each; the third bleeding obtained eighty cc. of blood and caused death of the rabbit.

The second rabbit was injected with five hundred mgs. of 10-HSA antigen. It was bled out only once on the tenth day after injection. Ninety cc. of blood were obtained, and the loss of blood caused the death of the rabbit. The sera were removed after clotting and refrigeration, and were stored with merthiolate. The antisera of the first rabbit were designated as 10-HSA-4a, 10-HSA-4b, and 10-HSA-4c; that of the second rabbit was designated as 10-HSA-5a.

Precipitation tests were carried out with the sera thus obtained, using as test antigens those compounds which had been run previously against 10-HSA sera. The protocols for these tests were the same as those used for the earlier precipitation reactions. Results of these experiments are shown in Table XIII. The reactions with DBA-Bov are omitted, since the non-specific precipitation by control sera lowered the value of this conjugate as a test antigen for haptene activity.

These sera compared favorably with the 10-HSA sera previously obtained, and served to confirm the haptene activity of 1,2-benzanthracene. Although the amount of precipitate in all of these tests on the new sera was slightly less than the average obtained for the old 10-HSA sera, they still showed far greater activity than the BP-HSA and DBA-HSA sera. There was little difference in the precipitation reactions of the two sera, despite the fact that rabbit no. 4 had received one hundred and fifty mgs. less antigen than did rabbit no. 5. The three sera obtained on consecutive days from the same rabbit did not differ to a significant degree in their precipitation reactions.

TABLE XIII

PRECIPITATION TESTS USING NEW SERA AGAINST 10-HSA

Antigen	Antiserum	Antigen dil. showing max. ppt.	Reading of maximum ppt.	Highest dil. showing ppt.
10-HSA	10-HSA-4a	1-600, 800	+±	1-3000
"	4b	600	++	3200
"	4c	600	+±	3000
"	5a	800, 1000	++	3000
BP-HSA	10-HSA-4a	1400	++	1600 *
"	4b	1200, 1400	++	1600 *
"	4c	1200	+±	1600 *
"	5a	1200, 1400	++	1600 *
DBA-HSA	10-HSA-4a	200	±	800
"	4b	200	+	1000
"	4c	200, 400	+	1000
"	5a	200, 400	+	1200
HSA	10-HSA-4c	800	+	1600 *
"	5a	600	++	1600 *
10-Bov	10-HSA-4c	32	+	256
"	5a	16	±	256
BP-Bov	10-HSA-4a	4	±	16
"	4b	8	±	32
"	4c	4	+	32
"	5a	1	±	8

* -highest dilution tested

E. Inhibition reactions

The second method for demonstrating serological specificity used by Landsteiner and his coworkers (35, 34, 40) was the inhibition of precipitation reactions by the haptene, or simple derivatives thereof. The inhibitors did not precipitate with antisera, but did prevent the formation of antigen-antibody precipitates. This method has gained rather wide use in immunology as an aid in determining the specificity of serological reactions, since it eliminates the possibility of influence of protein structures on the precipitation reactions.

The application of the inhibition method in studying the haptene activity of the hydrocarbons was limited to use of derivatives, since the hydrocarbons themselves are insoluble in saline solution. One type of moderately water-soluble derivative of the hydrocarbons was prepared by Dr. Creech by means of digestion of hydrocarbon-protein conjugates with pepsin, trypsin, and erepsin. The products of these digestions were presumed to consist of small polypeptides, some of which were linked to hydrocarbon groups. The following proteins were hydrolyzed by this method: Horse serum albumin, 3,4-benzpyrenyl-5-carbamido-horse serum albumin, 1,2-benzanthryl-10-carbamido-horse serum albumin, and 1,2,5,6-dibenzanthryl-9-carbamido-horse serum albumin.

The digests were tested for their ability to inhibit the precipitation reactions. They were diluted serially up to 1-16 with saline solution. One-tenth cc. of undiluted antiserum was mixed with one-tenth cc. of each dilution of digest, and the two incubated at 46°C. for one hour. Then the optimum concentration of the test antigen was added to each tube, which was subsequently reincubated for one hour and placed in the refrigerator overnight. Control tubes were set up by using only antiserum and antigen, and replacing the digest with an equal volume of saline solution. A second set of control tubes omitted the antigen from the inhibitor-antiserum system.

The results of the inhibition tests, which are shown in Table XIV, were confused by the erratic behavior of the digest made from DBA-HSA, and by the DBA-Bov antigen. In all cases, the antisera yielded precipitates with this DBA-HSA digest in the absence of the test antigens. Therefore, more precipitate was formed when the

digest was present with the antigen-antibody system than when it was absent. It should be recalled that the DBA-Bov antigen had previously been guilty of non-specific precipitation in the presence of control sera. Apparently, the low solubility of the 1,2,5,6-dibenzanthracene (i.e. DBA) conjugates limited their value in demonstrating haptene activity and specificity.

On the other hand, the 1,2-benzanthracene (i.e. "10") and the 3,4-benzpyrene (i.e. "BP") conjugates, when digested with enzymes, yielded inhibitors which did not form precipitates with the antisera, and were effective in inhibiting precipitation reactions. The 10-digest completely inhibited the reactions of the 10-HSA sera with the 10-Bov antigen, and partially inhibited the reactions with BP-Bov and 10-HSA antigens. The inhibition by the 10-digest of the precipitation with the DBA-Bov test antigen did not appear logical, since this precipitation had been considered comparatively non-specific.

The BP-digest partially inhibited the reaction of BP-HSA antisera with BP-Bov antigen, and of one 10-HSA serum with 10-Bov antigen. These cross inhibition reactions indicate that the haptene activity is not completely specific; if it were, only reactions with homologous hydrocarbons would have been inhibited.

These inhibition reactions confirmed the conclusions drawn from the precipitation reactions, i.e. a) that 1,2-benzanthracene possesses the strongest haptene group activity of the three hydrocarbons tested; b) that cross reactions do occur in the haptene activity of these hydrocarbons, indicating lack of complete specificity; c) that 1,2,5,6-dibenzanthracene derivatives are of limited value in the serological tests because of their tendency to non-specific precipitation.

TABLE XIV

INHIBITION REACTIONS USING ENZYMATIC DIGESTS OF HORSE SERUM ALBUMIN AND HYDROCARBON-HORSE SERUM ALBUMIN CONJUGATES AS TEST INHIBITORS

Antiserum	Antigen	Inhibitor: digest of HSA	10-HSA	BP-HSA	DBA-HSA
HSA-2b	HSA	O			
"	DBA-Bov	O	O	O	I
10-HSA-2a	10-HSA		+		
" 2a	10-Bov	O	+++	+	I
" 2a	DBA-Bov				I
" 3a	10-HSA		+		
" 3a	10-Bov	O	+++		
" 4c	DBA-Bov	O	++	O	I
" 5a	DBA-Bov	O	++	O	I
BP-HSA-1a	BP-Bov	O	O	+	I
" 2b	BP-Bov	O	+	+	I
2b	DBA-Bov	O	I	I	I
DBA-HSA-1a	DBA-Bov	I	I	I	I
" 2a	DBA-Bov				I
" 3a	DBA-Bov	I	I	I	I
Normal	DBA-Bov	O	O	O	I

O -no inhibition
+ -slight inhibition
++ -moderate inhibition
+++ -marked or complete inhibition
I -increased precipitation

F. Quantitative studies

It was apparent from the results of the precipitation reactions described in the preceding sections that a high-end titer of any antigen cannot be correlated with a large amount of precipitate at the

zone of optimum proportions, nor is it indicative of strong haptene group activity. Comparison of maximum precipitation readings was of value only if the same amount of antiserum was used for each test. Even if this condition was satisfied, the optimum antigen-antibody ratio and the amount of precipitation at this optimum ratio varied so much from serum to serum, that only rough comparisons of antibody potency were valid. A standard accurate method was therefore necessary for evaluation of the strength of the antigen-antibody reactions of these sera.

The method chosen to fulfill this requirement was the quantitative determination of the amount of precipitate, when the serum and antigen were mixed in optimum proportions to give maximum precipitation. This method has been used by Hiedelberger and Kendall for carbohydrate antigens (24, 25), and by Marrack and Smith for protein antigens (43). The precipitates formed in these reactions consist almost entirely of antibody, which is a gamma serum globulin. Since the globulin is protein, nitrogen analyses indicate the amount of protein in the precipitate. In the case of protein antigens, a small amount of the nitrogen in the precipitate is contributed by the antigen. However, the ratio of antibody nitrogen is so large that the latter figure does not alter the significance of the results.

The following procedure was used in determining the protein content of the precipitates. Undiluted antiserum (five-tenths or one or two cc.) was pipetted into each of three fifteen-cc. centrifuge tubes. Dilutions of antigen were added to the serum. In the first tube, the amount of antigen was directly proportional (in relation to

the quantity of serum) to the least amount of antigen shown by the precipitation tests to be in excess of the optimum ratio of antigen and antibody. In the second tube, the optimum amount of antigen was added. The third tube contained the most amount of antigen calculated to give a ratio of slight excess of antibody. In some cases where two or more tubes showed the maximum amount of precipitate, by visual observation, all of these antigen dilutions were run, as well as the tubes of excess antigen and antibody. This was done to determine quantitatively the dilution of optimum precipitation.

The tubes were placed in the 46°C. incubator, for one hour, then refrigerated overnight. After refrigeration, the precipitates were centrifuged, and the supernatant fluid was removed by pipette. The precipitates were then washed three times with saline solution, by stirring and shaking the precipitate with a few cc. of saline, re-centrifuging, and removing the clear supernatant. This procedure removed uncombined antibody, uncombined antigen, and the other proteins of the antiserum; the precipitate itself does not dissolve in the saline solution (4).

Quantitative micro-Kjeldahl analyses for the amount of nitrogen were then carried out. The determinations were made by Mr. Max Tryon and Mr. Edward Walton of the Chemistry Department of the University of Maryland. The method for nitrogen analysis was a modification of the microanalytical method of Pregl (50). The precipitate was first digested with one cc. of sulfuric acid until clear. The digest was then run into a microKjeldahl distillation apparatus, manufactured by the Standard Scientific Glass Company. Ten cc. of sodium hydroxide were added to the sample, and the ammonia formed was steam-distilled into a receiver

TABLE XV

MILLIGRAMS OF PROTEIN PRECIPITATED PER ONE CC. OF ANTISERUM

Antiserum	Antigen	Mgs. of antigen added per 1 cc. of antiserum															
		10.0	5.0	4.0	2.5	1.6	1.2	0.80	0.60	0.40	0.20	0.15	0.10	0.09	0.05	0.04	0.025
HSA-1a	HSA							0.32					1.80		1.54		1.02
" 1a	BP-HSA			0.39				0.27		0.41	0.43						
" 2b	HSA							0.30			1.81		1.06		0.74		0.35
BP-HSA-1a	BP-HSA					0.40				0.82	1.36		0.69				
" 2b	BP-Bov		0.20														
" 2b	DBA-Bov	0.20*															
10-HSA-1a	10-HSA									0.68			0.76		0.55		0.23
" 1a	10-Bov				0.34				0.66			0.48					
" 1a	BP-Bov						0.43										
" 2a	DBA-Bov	0.25*															
" 5a	10-HSA									0.51			0.49			0.29	
" 5a	HSA									0.28				0.34			0.46
DBA-HSA-1a	DBA-HSA					1.08		1.21		0.97	0.73						
" 2a	DBA-Bov	0.10*															

* -value after correction for amount of precipitate with normal serum.
Underscoring indicates maximum precipitate.

containing ten cc. of a four percent boric acid solution to which methyl red had been added. The use of boric acid in place of the standard method of absorption of distillate in hydrochloric acid has been recommended by Wagner, et al. (17, 45). The distillate in the receiving flask was titrated with standard sulfuric acid to the color of a control boric acid solution. The amount of nitrogen in milligrams in the precipitate was calculated from the amount of sulfuric acid used. This figure was then multiplied by 6.25 to give the number of milligrams of protein in the precipitate.

The results of these quantitative studies are shown in Table XV. The amounts of antigen and the precipitate were based on reactions with one cc. of antiserum. If a different quantity of antiserum was used in setting up a precipitation test, the figures in the table were those calculated from the experimental data for one cc. of antiserum.

The optimum amounts of antigen for maximum precipitation (underscored in the table) as determined by these quantitative analyses agreed with the values obtained by visual observation, with a few exceptions, namely the precipitation reactions of 10-HSA-5a sera and the reactions of HSA-1a serum with BP-HSA. In the other cases, however, the maximum precipitation was found to be by quantitative analysis near the center of the range of antigen dilutions, or at the antigen dilution predicted by visual observations.

The data were not sufficiently complete to draw definite conclusions concerning the value of this method for antibody potency. Because of the number of serological tests that had been carried out, sufficient quantities of the strongly precipitating antisera were not

available for thorough quantitative studies. However, a few general comments appear to be warranted by the results which were obtained.

First, it can be seen that the amount of precipitate formed on reaction of antisera against the conjugates with the homologous antigens was in no case as high as that formed on reaction of sera against horse serum albumin with their homologous antigen. This indicated a definite loss in protein antigenicity on conjugation; the extent of this loss might be comparable to the decrease in the amount of precipitate formed with equal quantities of antigen at the optimum zone. For instance, for the system of horse serum albumin and HSA-la serum, the precipitate contained 1.80 mgs. of protein; for the system of 1,2-benzanthryl-10-carbamido-horse serum albumin and 10-HSA-la serum, there was 0.76 mg. of protein in the precipitate. Thus, the antigenicity may have been decreased approximately one-half on conjugation.

A second deduction made after observation of the results was that the most precipitate was formed with homologous antigen. As the test antigen diverged more from the homologous antigen, greater quantities of test antigen were required at the optimum zone, with smaller quantities of precipitate resulting. This statement can be verified by observing in Table XV, where the top line in each serum group denoted reactions with homologous antigen. Reactions with non-homologous conjugated antigens showed a shift to the left side of the table, i.e to the region of higher antigen concentration. Concurrent with this shift in the amount of antigen, a drop in the amount of precipitate was evident. A comparison of the maximum precipitates using antiserum 10-HSA-la with three different antigens will serve to illustrate the

general trend of the reactions. The most precipitate was formed with the homologous 10-HSA antigen. When the test antigen was 10-Bov, which contained the same hydrocarbon but a different protein, the use of six times as much antigen produced slightly less precipitate. When BP-Bov, a completely heterologous antigen was used, the amount of antigen required rose to twelve times that required by the homologous antigen, yet only slightly more than one-half the amount of precipitate was formed.

Evaluation of the comparative haptene activity of the three hydrocarbons on the basis of the amount of precipitated protein confirmed the conclusion, reached after visual observations, that 1,2-benzanthracene was the most efficient of the three. Examination of the results in Table XV showed that 10-HSA sera formed heavier precipitates with all heterologous antigens tested than did DBA-HSA or BP-HSA antisera.

EXPERIMENTAL

PART II

I. PURPOSE

The experiments described in Part I had shown that only one of the three hydrocarbons tested, ie. 1,2-benzanthracene, possessed strong haptene group activity. The other two hydrocarbons, 3,4-benzpyrene and 1,2,5,6-dibenzanthracene, exhibited less effective antigenicity in the production of antisera directed against these hydrocarbon haptene groups. Therefore, it was considered desirable that further experimentation on the immunological behavior of the three hydrocarbons be limited to antisera developed by injection of serum albumin conjugates with 1,2-benzanthracene only.

The preliminary studies on the quantitative analysis of precipitates had indicated that this method would be of value in determining the loss of antigenic properties of the albumin on conjugation with hydrocarbon, and the degree of immunological cross-reactions among these hydrocarbons. However, the preliminary quantitative experiments had not been sufficiently extensive to establish with certainty either of these phenomena, since only a few sera of high antibody content were available for testing.

A comprehensive study of sera against serum albumin conjugates of 1,2-benzanthracene was therefore undertaken for the purpose of determining, by quantitative methods, the pattern of serological relationships with homologous and heterologous antigens.

II. ANTIGENS

Two serum albumin conjugates of 1,2-benzanthracene were prepared for use as immunization antigens, namely 1,2-benzanthryl-10-carbamido-

human serum albumin and 1,2-benzanthryl-10-carbamido-horse serum albumin.

Human serum albumin was obtained from the laboratory of Dr. Edwin J. Cohn, of the Harvard Medical School. Reaction of the albumin with 1,2-benzanthryl-10-isocyanate resulted in a conjugate containing twenty-nine hydrocarbon groups per molecule of albumin. The conjugate was used for injection and testing in a concentration of nine milligrams per cc. of saline solution.

The horse serum albumin was isolated in this laboratory by means of fractional ammonium sulfate precipitation from horse plasma. The fractionation of the components of horse plasma was carried out by salting out the proteins at different concentrations of ammonium sulfate, according to the method described by McMeekin (44). The ammonium sulfate was placed in a rotating cellophane bag, and allowed to diffuse into the protein solution. The apparatus is sketched in Figure 7. The content of salt in the protein solution could be determined by the density of the solution, after correction for the amount of protein present and for the temperature. The corrected density reading then corresponded to a definite concentration of ammonium sulfate.

Five and three-tenths liters of horse blood was collected in a citrate-saline solution; the red blood corpuscles were allowed to settle, and the supernatant plasma was removed by suction. The plasma (three thousand one hundred and fifty cc.) was diluted with three thousand cc. of 2M ammonium sulfate to give a concentration of 0.94M salt. Thereafter, the following fractions were removed in series, by placing the calculated amount of ammonium sulfate in the cellophane

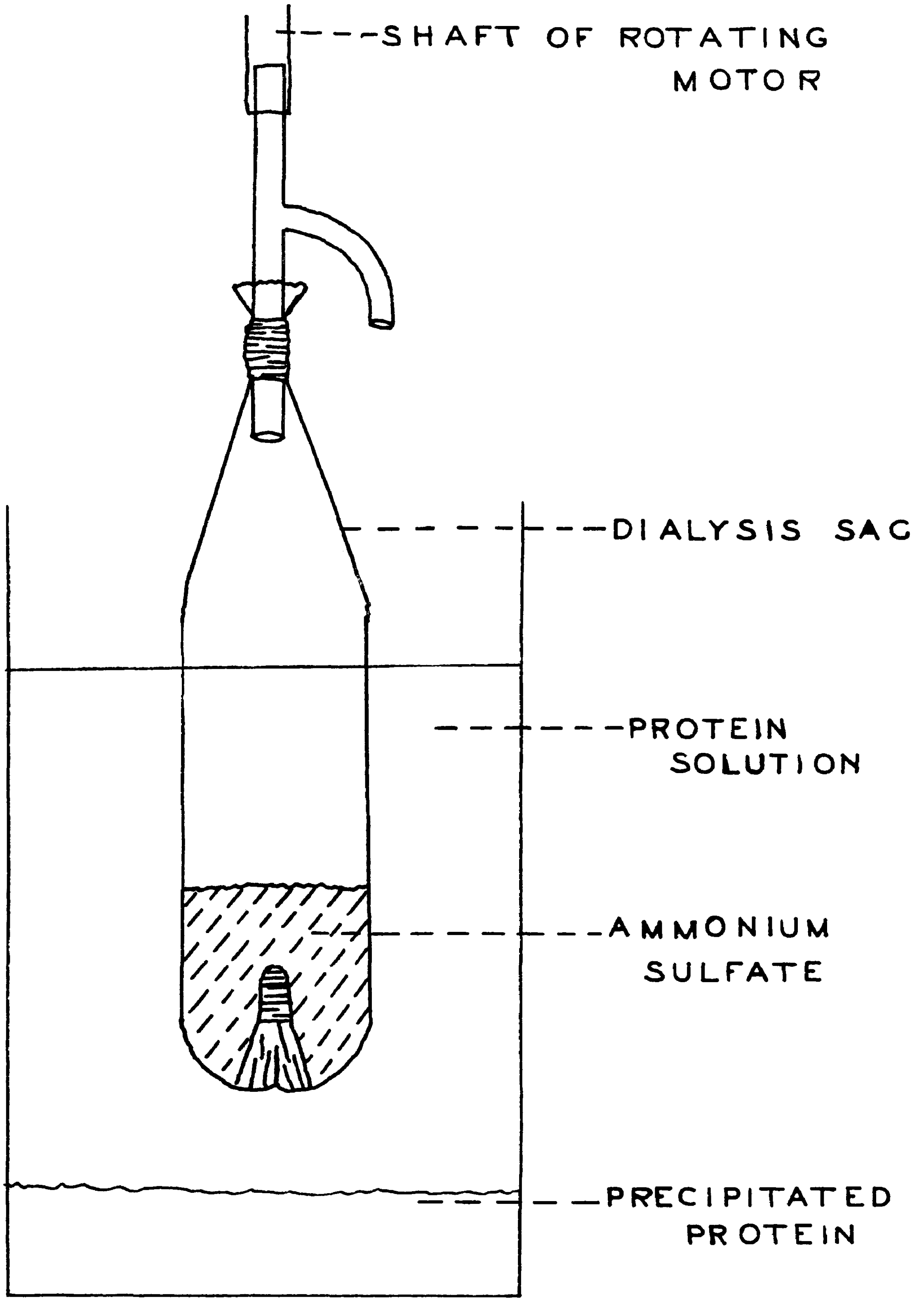


Fig. 7 - [illegible]

bag, rotating it in the protein solution for about twenty-eight hours or more and allowing the precipitated protein to settle for about twenty hours. All of these operations were carried out in the refrigerator.

Concentration of salt	Probable content of fraction removed
At 0.96 M	fibrinogen
1.29 M	gamma-globulin
2.05 M	alpha- and beta-globulin
2.49 M	albumin
2.71 M	albumin

The albumin precipitates were dialyzed against tap and distilled water at 7°C. for forty-eight hours to remove the ammonium sulfate. The solution of protein, presumably largely albumin, was brought to a concentration of 2.0 M ammonium sulfate, and the small amount of precipitate, mainly globulin, was discarded. The concentration of salt was then raised to 2.31 M and a large amount of precipitate was formed. Microscopic examination showed large numbers of typical horse serum albumin crystals:

This crystalline precipitate was subjected to two recrystallizations.

To the supernatant from the precipitation at 2.31 M salt was added ammonium sulfate to give a concentration of 2.55 M salt. Heavy precipitation of the albumin occurred, giving a completely crystalline product. This was dialyzed against tap and distilled water at 7°C. for forty-eight hours. Calculation of the micro-Kjeldahl data for protein content of the albumin solution showed fifteen and four-tenths grams of protein in the three hundred and fifty cc. of solution;

however, it was later found that the calculation was erroneous, and that the actual amount of crystalline albumin obtained in this preparation was thirty-eight and five-tenths grams.

Several other fractionations of the supernatants of the two major albumin precipitations were carried out, but these yielded at best only partially crystalline products. The fraction obtained at 2.55 M ammonium sulfate was selected for injection, and for coupling with 1,2-benzanthracene.

The coupling reaction between horse serum albumin and 1,2-benzanthryl-10-isocyanate was carried out by Dr. Creech according to the procedure described in Part I, section II. The resulting horse serum albumin conjugate of the hydrocarbon contained sixteen groups per albumin molecule, in comparison with the thirty-eight group conjugate used in the previous experiments. This decrease in the number of hydrocarbon groups was due to the error in computing the amount of crystalline horse serum albumin. Since there was two and one-half times more protein present than originally calculated, the number of hydrocarbon groups on each protein molecule was correspondingly reduced approximately two and one-half times. The conjugate was dissolved in saline solution; the concentration of protein was ten milligrams per cc. of solution.

Unmodified horse serum albumin and human serum albumin were also used as antigens for immunization. Each protein was dissolved in saline solution to give a concentration of ten milligrams of protein per cc. All four antigens were stored with merthiolate as preservative in the refrigerator in sterile rubber-capped bottles.

III. IMMUNIZATION SERIES

Twelve albino rabbits were used for immunization. They were obtained from a different dealer in laboratory stock than those used in the earlier experiments. Four were male, and eight were female. The rabbits were fed Purina chow, occasional greens, and water.

The animals were separated into four groups, differentiated on the basis of antigen received. Table XVI shows the groups into which the rabbits were divided.

TABLE XVI

RABBIT GROUPS ON BASIS OF ANTIGEN RECEIVED

Group No.	No. of Rabbits	Antigen
I	2	Horse serum albumin (HSA)
II	4	1,2-Benzanthryl-10-carbamido-horse serum albumin (10-HSA)
III	2	Human serum albumin (USA)
IV	4	1,2-Benzanthryl-10-carbamido-human serum albumin (10-USA)

The rabbits were injected three times per week for five weeks with the appropriate antigen. Approximately forty-five milligrams per injection were given intraperitoneally for three alternate weeks. Approximately ten milligrams per injection were given subcutaneously for the other two weeks. A total of approximately four hundred and fifty milligrams was injected into each of ten rabbits. Since difficulties in the coupling reaction between 1,2-benzanthracene and human serum albumin had resulted in a small yield of 10-USA conjugate, insufficient antigen was available for immunization of all four rabbits of the 10-USA

group with four hundred and fifty milligrams each. It was therefore decided to cut down the antigen dose of two of these rabbits to two hundred and twenty-five milligrams of 10-USA.

In an attempt to compensate for this reduction in immunizing antigen, these two rabbits were also injected intraperitoneally with one cc. of a sterile solution of Wilson's Solubilized Liver Fraction L, containing fifty milligrams of protein per cc., with each injection of antigen. Milone, Fesare, and Veitch (46) had shown that injection of a soluble liver extract in conjunction with immunization of rabbits with a Salmonella schottmülleri bacterin enhanced the production of antibodies, as demonstrated by agglutination and mouse protection tests.

TABLE XVII

TOTAL AMOUNT OF ANTIGEN AND LIVER EXTRACT INJECTED IN SECOND SERIES OF IMMUNIZATION OF RABBITS

Rabbit no.	Antigen	Amt. of antigen injected	Amt. of liver extract injected	Designation of serum
3	HSA	450 mgs.		HSA-3a
10	HSA	449 mgs.		HSA-4a
11	10-HSA	450 mgs.		10-HSA-6a
31	10-HSA	458 mgs.		10-HSA-7a
27	10-HSA	450 mgs.	700 mgs.	10-HSA-8a
35	10-HSA	450 mgs.	700 mgs.	10-HSA-9a
15	USA	456 mgs.		USA-1a
50	USA	456 mgs.		USA-2a
4	10-USA	413.2 mgs.*		10-USA-1a
40	10-USA	312.4 mgs./		10-USA-2a
25	10-USA	228.6 mgs.	700 mgs.	10-USA-3a
48	10-USA	226.7 mgs.	700 mgs.	10-USA-4a

* -Last injection was not given because of lack of available antigen.

/ -Rabbit was not gaining weight, ate little food, and appeared scrawny, so the injection series was discontinued. The condition of the rabbit improved considerably after injections were stopped.

Two of the rabbits which were injected with four and fifty milligrams

of 1,2-benzanthryl-10-carbamido-horse serum albumin were also given the liver extract, in an effort to produce more potent sera against this antigen. The amount of antigen injected into each rabbit and the designation of the serum from each, are shown in Table XVII.

IV. BLEEDING PROCEDURE

Ten days after the last injection, each rabbit was bled from the ear, and the precipitin titer against homologous antigen determined. Since the titer in most cases appeared satisfactory, i.e. comparable to titers obtained in previous experiments, no additional antigen was injected. The rabbits were bled out seven to ten days after the preliminary ear bleeding. This final bleeding was accomplished by anaesthetizing the rabbit with ether, then bleeding from the heart with a gauge no. 18 needle and a fifty cc. syringe. The amount of blood obtained ranged from sixty-five to ninety cc. The blood was allowed to clot at room temperature for one hour, placed in the refrigerator overnight, and the serum was removed by centrifugation or pipette. The sera were stored in sterile bottles with a few drops of merthiolate as preservative.

V. SEROLOGICAL INVESTIGATIONS

Precipitation reactions of these twelve sera were conducted using both homologous and heterologous antigens. A thorough investigation of serological reactions was necessary before quantitative analyses of these reactions could be carried out, in order to determine the zone of maximum precipitation for each antigen-antibody reaction.

A. Methods

The procedure for carrying out these serological tests varied from the methods used in Part I as follows:

1) The test tubes used measured seven millimeters by fifty millimeters, instead of ten millimeters by seventy-five millimeters.

2) For homologous antigens, the serum was usually diluted in half with saline solution, and further diluted again by one-half if three zones of precipitation were not demonstrated with the larger quantity of serum. For heterologous antigens, the serum was used undiluted. Because of the viscosity of the serum and the narrowness of the tubes, the serum would not run to the bottom of the tubes when a pipette was used. Therefore, one-tenth cc. of diluted or undiluted serum was placed in the tubes by using a two cc. syringe and a one and a half inch, gauge no. 20 needle.

3) The volume of each antigen dilution was reduced from two-tenths cc. to one-tenth cc. so that the tests would require less antigen, especially of those conjugates which were available only in small quantity.

4) The antigen-antiserum mixture was incubated at room temperature, rather than at 46°C., primarily for convenience. Preliminary tests had shown that equal amounts of precipitate were formed when the tubes were incubated at 46°C. and at room temperature.

5) The tubes were read, after refrigeration, by light from a Spencer adjustable microscope lamp, with iris diaphragm. The lack of glare and the sharper images obtained made this light preferable to shaded incandescent light.

B. <u>Homologous serum albumins and 1,2-benzanthryl-10-carbamido-serum albumin conjugates as test antigens</u>

1. <u>With sera against unmodified proteins</u>

Each of the four sera against unmodified proteins was mixed with

the homologous antigen or with the 1,2-benzanthryl conjugate of that protein. This was done to determine whether conjugation with hydrocarbon limited the antigenicity of the serum albumins, as indicated by lighter precipitation when the conjugated protein rather than the unmodified protein was used for testing the sera. The results of these experiments are shown in Table XVIII.

TABLE XVIII

PRECIPITATION REACTIONS USING HOMOLOGOUS SERUM ALBUMINS AND 1,2-BENZANTHRYL-10-CARBAMIDO-SERUM ALBUMINS AS TEST ANTIGENS WITH SERA AGAINST UNMODIFIED SERUM ALBUMINS

Antiserum	Antigen	Dilution of serum	Antigen dil. of max. ppt.	Reading at maximum ppt.
HSA-3a	HSA	1 - 4	1-100	+±
HSA-3a	10-HSA	Undil.	1-8, 16	++±
HSA-4a	HSA	1 - 4	1-50	++++
HSA-4a	10-HSA	Undil.	1-4	++++
USA-1a	USA	1 - 2	1-200	±
USA-1a	10-USA	Undil.	Undil.	tr
USA-2a	USA	1 - 2	1-100	+++
USA-2a	10-USA	1 - 2	1-2	+

Comparison of the results with serum HSA-3a and both antigens showed that with conjugated horse serum albumin, four times as much antiserum and approximately ten times as much antigen were required to produce only slightly more precipitate than that obtained with unmodified horse serum albumin. Similar results were given by the other sera; much larger quantities of antiserum and of conjugated albumin were required in all cases to produce comparable, or even lower, maximum precipitation. Thus a decrease in reactivity of both albumins on conjugation with 1,2-benzanthracene was shown. It should also be noted that the decrease appeared to be more marked in the case of the human

serum albumin conjugate; the precipitates with these antigens were considerably lighter than those with unmodified human serum albumin.

For comparable quantities of antiserum and unmodified albumin, the sera against human serum albumin did not give as heavy precipitates as did sera against horse serum albumin. This indicated lower antigenic potency for human serum albumin than for horse serum albumin.

2. With sera against 1,2-benzanthryl-10-carbamido serum albumins.

The four sera against 1,2-benzanthryl-10-carbamido horse serum albumin, and the four sera against 1,2-benzanthryl-10-carbamido-human serum albumin were tested with the antigen used for injection, and with the unmodified albumin from which that conjugate was made.

Table XIX presents the results of these experiments, which showed that, in all cases, the antisera against the human serum albumin conjugate yielded a smaller amount of precipitate with homologous antigen than did sera against the horse serum albumin conjugate. This confirmed the results shown above on the lower antigenic potency of human serum albumin.

Reactions of all eight sera with the unmodified albumins were of lower intensity than with the hydrocarbon-albumin conjugates. Apparently, the modification of the albumins on conjugation extended to their ability to incite the formation of antibodies against the albumin moities of the conjugates, as well as their ability to form precipitates with antisera against the unmodified protein, as shown in Table XVII. The experiments with which these two tables are concerned confirmed the observations in Part I on the marked loss in antigenicity of the serum

TABLE XIX

PRECIPITATION REACTIONS USING HOMOLOGOUS SERUM ALBUMINS AND 1,2-BENZANTHRYL-10-CARBAMIDO-SERUM ALBUMINS AS TEST ANTIGENS WITH SERA AGAINST 1,2-BENZANTHRYL-10-CARBAMIDO-SERUM ALBUMINS

Antiserum	Antigen	Dilution of serum	Antigen dil. of max. ppt.	Reading at maximum ppt.
10-HSA-6a	HSA	1 - 2	1 - 100	+±
10-HSA-6a	10-HSA	1 - 2	1 - 50	++±
10-HSA-7a	HSA	Undil.	0	0
10-HSA-7a	10-HSA	Undil.	1 - 200	+++
10-HSA-8a	HSA	1 - 2	1 - 100	+
10-HSA-8a	10-HSA	Undil.	1 - 200	+++
10-HSA-9a	HSA	1 - 2	1 - 200	±
10-HSA-9a	10-HSA	Undil.	1 - 100	+++
10-USA-1a	USA	1 - 2	1 - 100	±
10-USA-1a	10-USA	1 - 4	1 - 50	+±
10-USA-2a	USA	1 - 2	1 - 400	±
10-USA-2a	10-USA	1 - 2	1 - 100	++
10-USA-3a	USA	1 - 2	1 - 400, 800	tr
10-USA-3a	10-USA	1 - 2	1 - 100	+±
10-USA-4a	USA	1 - 2	1 - 400	±
10-USA-4a	10-USA	1 - 4	1 - 50	±

albumins on conjugation with hydrocarbon groups through a carbamido linkage.

The last two sera in each group in Table XIX were obtained from rabbits which had received seven hundred milligrams each of the soluble liver extract. Comparison of the serological reactions indicated that this liver extract did not raise the antibody content of the sera, since there was no significantly greater precipitates with these sera than with those from rabbits which had not received the liver extract. The sera from the two rabbits which had received only half the usual

amount of antigen, i. e. sera 10-USA-3a and 10-USA-4a, showed weaker precipitation reactions than those sera from rabbits which had been injected with the full four hundred and fifty milligrams of antigen. Thus, the liver extract did not appear to influence the precipitin content of these sera.

C. Heterologous serum albumins as test antigens with all antisera

Preliminary to experiments demonstrating haptene activity, it was deemed necessary to test all antisera for their lack of reactivity with heterologous albumins, in order to make certain that later experiments with heterologous conjugated albumins would indicate only haptene activity.

The six antisera against horse serum albumin, or the 1,2-benzanthracene conjugate thereof, were mixed with human serum albumin and bovine serum albumin. The six antisera against human serum albumin, or the 1,2-benzanthracene conjugate thereof, were tested against horse serum albumin and bovine serum albumin. Eight of these twelve sera did not precipitate at all with the heterologous serum albumins. Two of the sera, namely 10-HSA-9a and 10-USA-3a, yielded precipitates read as "very faint trace" with a 1 - 4 dilution of bovine serum albumin; serum USA-2a yielded a precipitate read as "trace" with a 1-2 dilution of bovine serum albumin. These reactions were considered so slight as to be of negligible importance in influencing haptene group activity.

Serum HSA-4a gave fairly strong reactions with the heterologous albumins. When one-tenth cc. of serum (diluted 1 - 2) was mixed with one-tenth cc. of the optimum 1 - 4 dilution of human serum albumin, the amount of precipitation was recorded as "+±." When the same

amount of serum was mixed with one-tenth cc. of a 1 - 4 dilution of bovine serum albumin, the precipitate was read as "+." There was a possibility that the rabbit had been injected with human serum albumin by mistake and so had formed antibodies against that protein. Such an error could not explain the results with the bovine serum albumin, however, since it was not used for injection.

Therefore, it was decided to attempt a precipitin absorption test, to see if these two precipitations were caused by non-specific cross reactions, or by specific antibodies. One cc. of a 1 - 2 dilution of the HSA-4a antiserum was mixed with one cc. of a 1 - 25 dilution of horse serum albumin (optimum zone). The mixture was incubated two hours at room temperature, and kept forty hours in the refrigerator. The resulting heavy precipitate was removed by centrifuging, and the supernatant was decanted. Precipitation tests were then run with horse, human, and bovine serum albumins at optimum zones for the 1 - 4 dilution of antiserum. In no case was the amount of precipitation in the tests greater than "faint trace", when any of the three serum albumins were used.

Apparently, therefore, the absorption with horse serum albumin not only removed almost all of the antibodies against horse serum albumin, but also those giving reactions with human and bovine serum albumins. The cross reactions of this serum appear to be due to non-specific antibodies, rather than to individual antibodies against the heterologous serum albumins.

D. <u>Heterologous conjugated serum albumins as test antigens for haptene activity.</u>

As explained in Part I, tests for the antigenicity of the haptene

hydrocarbon groups were conducted by mixing antiserum against one hydrocarbon-serum albumin conjugate with a hydrocarbon conjugate of a different serum albumin. Since only two of the sera against hydrocarbon-serum albumin conjugates had formed but "very faint trace" reactions with bovine serum albumin, it was assumed that reactions of all eight sera with a hydrocarbon-bovine serum albumin conjugate would be due almost entirely to antibodies directed against the hydrocarbon group.

The sera prepared by immunization with 1,2-benzanthryl-10-carbamido-horse serum albumin were tested against solutions of 1,2-benzanthryl-10-carbamido-human serum albumin (twenty-nine hydrocarbon groups per molecule; nine milligrams of antigen protein per cc. of solution); 1,2-benzanthryl-10-carbamido-bovine serum albumin (two preparations: a) thirty-one hydrocarbon groups per molecule; five milligrams of antigen protein per cc. of solution, and b) fifteen hydrocarbon groups per molecule; ten milligrams of antigen protein per cc. of solution); and 3,4-benzpyrenyl-5-carbamido-bovine serum albumin (nineteen hydrocarbon groups per molecule; five milligrams of antigen protein per cc. of solution). A preparation of 1,2,5,6-dibenzanthryl-9-carbamido-bovine serum albumin (twenty hydrocarbon groups per molecule; ten milligrams of antigen protein per cc. of solution) was also used, and the reactions with antisera were corrected to control values. All of these antigens, except for 1,2-benzanthryl-10-carbamido-human serum albumin, had been prepared by Dr. Creech a few years previous to the tests. During the interval, the antigens were preserved as precipitates in half-saturated ammonium sulfate solution. They were prepared for serological use by dialysis for

three days against cold distilled water, and for a fourth day against cold saline solution. After dialysis, the antigen solutions were analysed by the microKjeldahl method for protein content and were then diluted with saline solution to concentrations of either five or ten milligrams of antigen protein per cc. of solution. The results of these experiments to demonstrate haptene activity of 1,2-benzanthracene are shown in Table XX.

TABLE XX

PRECIPITATION REACTIONS OF ANTISERA AGAINST 1,2-BENZANTHRYL-10-CARBAMIDO-HORSE SERUM ALBUMIN WITH HETEROLOGOUS HYDROCARBON SERUM ALBUMIN CONJUGATES

Antigen (with no. of hydrocarbon groups)	Antiserum	Antigen dil. of maximum ppt.	Reading at maximum ppt.
10-USA (29)	10-HSA-6a	1 - 16	+
"	" -7a	1 - 8	±
"	" -8a	1 - 16	+
"	" -9a	1 - 8	±
10-Bov (31)	10-HSA-6a	1 - 8, 16	±
"	" -7a	Undil.	±
"	" -8a	Undil.	tr
"	" -9a	1 - 1, 2	ftr
10-Bov (15)	10-HSA-6a	1 - 8	±
"	" -7a	1 - 2	tr
"	" -8a	Undil.	ftr
"	" -9a	1 - 2	tr
BP-Bov (19)	10-HSA-6a	Undil.	ftr
"	" -7a	-	-
"	" -8a	-	-
"	" -9a	-	-
DBA-Bov (20)	10-HSA-6a	1 - 16	ftr
"	" -7a	-	-
"	" -8a	-	-
"	" -9a	-	-

Fairly strong precipitation was exhibited by all antisera with 1,2-benzanthryl-10-carbamido-human serum albumin, indicating pronounced haptene activity of 1,2-benzanthracene. Individual differences among the sera were not great. On the other hand, the reactions with the 1,2-benzanthryl-10-carbamido-bovine serum albumin conjugate (10-Bov) varied with the sera; only two sera showed moderate precipitation. This difference in precipitation with the two antigens might be explained by the fact that the human serum albumin preparation (i.e. 10-USA) was only a few months old, whereas the bovine serum albumin conjugates had been prepared approximately three years previous to these experiments. Some denaturation of these antigens might have occurred during the interval, and might have been responsible for a decrease in ability to precipitate with antisera. Nevertheless, precipitation, though to a lesser degree, did occur, confirming the results on haptene activity with the other heterologous antigen, 1,2-benzanthryl-10-carbamido-human serum albumin.

Only one serum formed precipitates with either the 3,4-benzpyrenyl- or 1,2,5,6-dibenzanthryl-bovine serum albumin conjugates. The experiments with these antigens were repeated, with the same results. It was not known whether an alteration of the antigen had also occurred during storage. Since the only serum which did react with these two antigens, namely serum 10-HSA-6a, also showed the strongest reactions with the heterologous conjugates of 1,2-benzanthracene, it was possible that only serum of high antibody potency against the homologous hydrocarbon would also react with the heterologous hydrocarbons.

The four antisera against 1,2-benzanthryl-10-carbamido-human serum albumin were then tested against a wider spectrum of antigens, since

three hydrocarbon conjugates of horse serum albumin were heterologous to the antigen used for immunization. The concentration of the test antigen solutions varied between five and ten milligrams of antigen protein per cc. of solution. Results of these precipitation reactions, shown in Table XXI, indicated that the antisera against 1,2-benzanthryl-10-carbamido-human serum albumin were capable of precipitating with heterologous antigens whose similarity to the immunizing antigen was in the hydrocarbon groups attached to the protein.

Much stronger reactions were shown by these sera when they were mixed with antigens containing the same hydrocarbon as that involved in the immunization procedure, i.e. 1,2-benzanthracene. Comparison of the precipitates obtained by using sera against 10-USA and those against 10-HSA (from Tables XX and XXI) indicated that sera against either conjugate exhibited approximately the same ability to react with the homologous hydrocarbon. It had been observed previously (see Tables XVIII and XIX) that sera against the human serum albumin conjugate of 1,2-benzanthracene exhibited weaker precipitation reactions than did sera against the horse serum albumin conjugate when the test antigens used were the antigen used for immunization and the protein moiety thereof.

These sera against 1,2-benzanthryl-10-carbamido-human serum albumin showed occasional weak precipitation with conjugates of 3,4-benzpyrene. The reactions were not limited to the sera showing the strongest reactions with conjugates of the homologous hydrocarbon, since the differences among the sera in their reactions with homologous hydrocarbon were neither great nor uniform.

TABLE XXI

PRECIPITATION REACTIONS OF ANTISERA AGAINST 1,2-BENZANTHRYL-10-CARBAMIDO-HUMAN SERUM ALBUMIN WITH HETEROLOGOUS HYDROCARBON-SERUM ALBUMIN CONJUGATES

Antigen (with no. of hydrocarbon groups)	Antiserum	Antigen dil. of maximum ppt.	Reading at maximum ppt.
10-HSA (16)	10-USA-1a	1 - 16	±
"	" -2a	1 - 16, 32	±
"	" -3a	1 - 4	tr
"	" -4a	1 - 32, 64	±
10-HSA (38)	10-USA-1a	1 - 16	±
"	" -2a	1 - 16	±
"	" -3a	1 - 16	±
"	" -4a	1 - 8	tr
10-Bov (31)	10-USA-1a	1 - 16	+
"	" -2a	Undil.	tr
"	" -3a	Undil.	tr
"	" -4a	Undil.	tr
BP-HSA (19)	10-USA-1a	-	-
"	" -2a	-	-
"	" -3a	-	-
"	" -4a	1 - 16	tr
BP-Bov (19)	10-USA-1a	1 - 4	ftr
"	" -2a	-	-
"	" -3a	-	-
"	" -4a	1 - 16, 32	ftr
DBA-HSA (21)	10-USA-1a	Undil.	±
"	" -2a	Undil.	±
"	" -3a	Undil.	ftr
"	" -4a	Undil.	ftr
DBA-Bov (20)	10-USA-1a	1 - 8, 16, 32	±
"	" -3a	-	-

The reactions of these sera with the horse and bovine serum albumin conjugates of 1,2,5,6-dibenzanthracene were generally heavier than those with the conjugates of 3,4-benzpyrene.

One of these sera, namely 10-USA-1a, was tested with three antigens which had not been previously used in the serological investigations.

They were β-anthryl-horse serum albumin, 1,2-benzanthryl-3-carbamido-horse serum albumin, and 10-methyl 1,2-benzanthryl-3-carbamido-horse serum albumin. The first conjugate was a derivative of anthracene, which was conjugated with horse serum albumin by means of an isocyanate group at the β position on the hydrocarbon. The latter two compounds were prepared by conjugation of albumins with the hydrocarbons through an isocyanate group at a different site on the benzanthracene molecule that that used for previous experiments. These conjugates had been prepared by Dr. Creech. The concentration of the first antigen was ten milligrams of protein per cc. of solution, of the latter two, five milligrams. Only a "very faint trace" precipitate was formed with the β-anthryl-horse serum albumin, whereas "trace" precipitates were formed with the derivatives of 1,2-benzanthracene, at the zones of maximum precipitation.

E. Inhibition reactions

Because of their insolubility, the hydrocarbons themselves could not be used to inhibit the reactions presumed to be due to haptene activity. In the previous experiments, enzymatic hydrolysates of hydrocarbon-horse serum albumin conjugates were used as inhibitors. These were not entirely satisfactory, because of their low hydrocarbon content and limited solubility.

For the experiments described below, certain amino acid derivatives of 1,2-benzanthracene were prepared by Mr. Larry Q. Green, formerly of the Chemistry Department of the University of Maryland. Since the hydrocarbon derivatives of glycine and ϵ-aminocaproic acid had a limited solubility in water, it was expected that conjugation of the hydrocarbons

with synthetic di- and tripeptides would give compounds having a greater solubility in water and consequently a greater usefulness in the inhibitory tests. The derivatives were only partially soluble in a dilute solution containing sodium carbonate and sodium bicarbonate; a few drops of dioxane were added to facilitate solution. The pH of the solution was then adjusted to 7.5 with N/10 HCl. The final concentration of the amino acid derivatives was considerably lower than that of the albumin derivatives used in previous serological tests. These amino acid derivatives, and the concentration of each per cc. of solution are listed as follows:

Derivative	Mgs./ cc.
1,2-benzanthryl-10-carbamido-aceto-glycine.............	4.44
1,2-benzanthryl-10-carbamido-aceto-glycyl-glycine......	1.20
α(1,2-benzanthryl-10-carbamido-)-glutaric acid........	0.72
ε (1,2-benzanthryl-10-carbamido-)-caproic acid.........	0.85

As a control for the inhibition tests, glycyl-glycine was used at a concentration of five and four-hundredths milligrams per cc. of solution.

The following method was used to detect inhibition of precipitation reactions by these derivatives of 1,2-benzanthracene. Four test tubes, each containing one-tenth cc. of antiserum, were prepared. Solutions of the amino acid derivatives were each diluted serially: 1 - 2, 1 - 4, 1 - 8. To the first tube of antiserum, one-tenth cc. of undiluted solution was added; to the second tube of antiserum, one-tenth cc. of the 1 - 2 dilution; to the third, one-tenth cc. of the 1 - 4 dilution; to the fourth, one-tenth cc. of the 1 - 8 dilution. Five such series, one for each amino acid derivative, were prepared for each serum tested, with the exception of 10-USA-4a, of which only a small amount of serum

was available. The two solutions were mixed and incubated at room temperature for one hour.

At the end of this incubation period, the optimum dilution of antigen was added and mixed thoroughly with the contents of the tubes. For sera against 1,2-benzanthryl-10-carbamido-horse serum albumin, the antigen added was 1,2-benzanthryl-10-carbamido-human serum albumin. In the case of sera against the human serum albumin conjugate, the horse serum conjugate was used as the test antigen. A control tube of antiserum and antigen, plus one-tenth cc. of saline, was also set up. The tubes were reincubated for one hour at room temperature, and then were placed in the refrigerator. The same procedure was used to set up an antiserum - amino acid derivative - homologous antigen system for each serum tested.

The results of these inhibition reactions are summarized in Table XXII. The glycyl-glycine did not inhibit any of the reactions tested. There was inhibition by all four 1,2-benzanthryl-derivatives of the reactions of the sera with heterologous antigens, with the exception of the aceto-glycine derivative on the reaction between 10-HSA-9a serum and 10-USA antigen. The inhibition was marked in the case of the ε-amino caproic acid derivative, and slight or moderate with the other compounds.

When homologous antigens were used, there was very slight inhibition of the precipitation by the ε-amino caproic acid and aceto-glycyl-clycine derivatives.

TABLE XXII

1,2-BENZANTHRYL-10-CARBAMIDO-AMINO ACID AND PEPTIDE CONJUGATES AS INHIBITORS OF PRECIPITATION REACTIONS

		1,2-benzanthryl-10-carbamido-				
Antiserum	Antigen	aceto-glycine	aceto-glycyl-glycine	α-(10)-glutaric acid	ε-(10)-caproic acid	glycyl-glycine
10-HSA-8a	10-HSA	0	±	0	±	0
" -8a	10-USA	++	++	+	+++	0
10-HSA-9a	10-HSA	0	±	±	±	0
" -9a	10-USA	0	+	+	+++	0
10-USA-1a	10-USA	0	±	0	±	0
" -1a	10-HSA	+	+	+	++	0
10-USA-4a	10-HSA			+	+++	

0 -no inhibition
± -very slight inhibition
+ -slight inhibition
++ -moderate inhibition
+++ -marked inhibition

VI. QUANTITATIVE STUDIES

Another method of determining serological activity of the antisera was used in the previous experiments. This method involved quantitative analysis of precipitates at the optimum ratio of antigen and antibody. Since the value obtained could be established in terms of a single standard, i.e. one cc. of antiserum, the various precipitation reactions tested could be compared with accuracy greater than that possible by visual observation of precipitation reactions. The quantity of each antigen-antibody precipitate indicated the degree of reactivity of the serum with that particular antigen.

The procedure used was essentially the same as that in Part I. Three or more tubes of antigen-antiserum mixtures were prepared, one tube of which contained the optimum ratio of the two components. Two

tubes each contained an excess of one component; in one case antigen, in the other, antiserum. As the investigations progressed, it became obvious that insufficient quantities of antisera would make it impossible to set up three tubes for each antiserum against each antigen. Therefore, when necessary, the optimum ratio mixture was the only one prepared. Occasionally, if the quantity of serum was adequate, one tube of a mixture containing excess component was also set up.

The mixtures were incubated at room temperature for two hours, and refrigerated overnight. The precipitate was centrifuged, and washed with approximately two cc. of cold saline solution. The washing was carried out by suspension of the precipitate in the saline solution and centrifugation at forty-five hundred R.P.M. in a small angle-head centrifuge in the refrigerator. The supernatant liquid was then removed by capillary pipette. Three such washings were carried out.

The precipitate was then mixed with one cc. of concentrated sulfuric acid, and subjected to digestion until clear. The analysis for nitrogen content of the digest was carried out by the author, in the laboratories of the Department of Bacteriology. The amount of protein in the precipitates was then calculated from the nitrogen analysis. The results of the analyses are shown in Tables XXIII and XXIV.

Table XXIII is concerned with the quantity of precipitate when sera against horse serum albumin, or 1,2-benzanthryl-10-carbamido-horse serum albumin, were tested with homologous and heterologous antigens. The reactions with homologous antigen are shown by the first line of the group of antigens tested with each serum. For all

TABLE XXIII

MILLIGRAMS OF PROTEIN PRECIPITATED PER ONE CC. OF ANTISERUM AGAINST HSA AND 10-HSA

		Milligrams of antigen																				
Antiserum	Antigen	10.00	5.00	4.00	2.50	2.25	2.00	1.25	1.125	1.10	1.00	0.80	0.[illegible]	0.563	0.55	0.40	0.313	0.281	0.275	0.20	0.156	0.10	0.05	0.025
HSA-3a	HSA											0.63				<u>1.20</u>				0.95				
" -3a	10-HSA				0.52			<u>1.35</u>					1.09											
HSA-4a	HSA			3.00								<u>7.24</u>≠				<u>4.10</u>≠								
" -4a	10-HSA		2.02		<u>4.58</u>			3.90																
10-HSA-6a	10-HSA						1.18									<u>2.00</u>				1.50				
" -6a	HSA																			<u>0.60</u>				
" -6a	10-USA									0.45					<u>0.58</u>≠				0.32					
" -6a	10-Bov												0.32≠				<u>0.35</u>≠							
" -6a	BP-Bov	<u>0.12</u>																						
10-HSA-7a	10-HSA										1.10											1.18	<u>1.32</u>	1.06
" -7a	10-USA								<u>0.30</u>															
10-HSA-8a	10-HSA										1.12											<u>1.74</u>	0.72	
" -8a	HSA																			<u>0.39</u>	0.31			
" -8a	10-USA								0.22					<u>0.34</u>				0.20						
10-HSA-9a	10-HSA										0.68											<u>1.04</u>	1.00	0.82
" -9a	HSA																			0.31	<u>0.38</u>		0.25	
" -9a	10-USA						0.31		<u>0.34</u>					0.23										

Underlining indicates maximum ppt.
≠ indicates average of two determinations

TABLE XXIV

MILLIGRAMS OF PROTEIN PRECIPITATED [illegible] CC. OF ANTISERUM AGAINST USA AND 10-USA

Antiserum	Antigen	40.00	17.6	8.80	5.00	4.40	4.00	3.60	1.80	1.25	0.72	0.625	0.40	0.36	0.313	0.20	0.18	0.156	0.10	0.09
		Milligrams of antigen																		
USA-1a	USA												0.90			**2.04**			1.90	
" -1a	10-USA		0.27	**0.33**																
10-USA-1a	10-USA					[illegible].94					**1.42**			1.24						
" -1a	USA															**0.33**				
" -1a	10-HSA (16)									**0.26≠**		0.23								
" -1a	10-HSA (38)											**0.24**								
" -1a	10-Bov											0.19			**0.34≠**			0.30		
" -1a	BP-HSA	**0.17**																		
" -1a	BP-Bov				**0.19**															
" -1a	DBA-HSA	**0.22**																		
" -1a	DBA-Bov									**0.12**										
10-USA-2a	10-USA								0.76								**1.08**		0	0.76
" -2a	USA															**0.33**				
" -2a	10-HSA (16)											**0.14**								
10-USA-3a	10-USA								0.20								**0.60**			0.50
" -3a	10-HSA (38)											**0.14**								
10-USA-4a	10-USA							**0.51**			0.40									
" -4a	USA																			**0.38**
" -4a											0.17				**0.20**					

Underlining indicated maximum ppt.

≠ indicates average of two determinations

the sera, except serum HSA-3a, the largest amount of precipitate at the optimum ratio was formed when this antigen was used. However, when the test antigen was heterologous to that used for immunization, the amount of precipitate dropped rather sharply.

In the case of serum HSA-3a, there was slightly more precipitate with the heterologous antigen 10-HSA than with the homologous antigen HSA. However, approximately three times as much heterologous antigen was required to produce the heavier precipitate.

The precipitates formed with serum HSA-4a were heavier than those of any other serum. This was the serum which reacted strongly with the heterologous antigens human serum albumin and bovine serum albumin. When the conjugated antigen, 10-HSA, was used as test antigen with this serum, the amount of precipitate was considerably less than that formed with the homologous protein, although approximately three times as much antigen was used.

The four sera against the conjugate, 10-HSA, showed heavier precipitates with this antigen than with any other. Far less precipitate, one-fourth to one-third, was formed with the optimum concentration of horse serum albumin, although similar amounts of antigen were used.

When the conjugate 10-USA was the test antigen with the sera against 10-HSA, the amount of this heterologous antigen required at the optimum ratio was always greater than that of the homologous antigen 10-HSA. In the case of serum 10-HSA-6a, the increase in antigen was from 0.40 milligrams to only 0.55 milligrams, but with serum 10-HSA-9a the rise was from 0.10 milligrams to 1.125 milligrams, a tenfold increase. Concurrent with the rise in the amount of required

antigen, there was also for all sera a marked decrease in precipitation, as compared with the values using homologous antigen.

When the completely heterologous antigen, BP-Bov, was tested against serum 10-HSA-6a, the yield of precipitate was further reduced, and a much greater concentration of antigen was required.

Table XXIV shows the results with the antisera against human serum albumin, and against 1,2-benzanthryl-10-carbamido-human serum albumin. Serum USA-2a was not used for the quantitative studies because it yielded only slight precipitates with any of the antigens against which it was tested. Analyses of the reactions with serum USA-1a were similar to those with sera against horse serum albumin in that more conjugated albumin than unmodified albumin was required at the optimum zone. In this case, the use of forty times as much conjugated antigen yielded only one-sixth the amount of precipitate. The increase in required antigen and the decreased yield of precipitate with the conjugated human serum albumin were much greater than with the conjugated horse serum albumin.

The four sera against 10-USA all gave greater precipitates with the homologous conjugate than with any other antigen. The rabbits from which sera 10-USA-3a and 10-USA-4a were obtained had received less antigen than the other two rabbits; the amount of precipitation of these two sera with the homologous antigen was approximately one-half that of the 10-USA-1a and 10-USA-2a sera.

When human serum albumin was used as the test antigen with three of the 10-USA sera, the amount of antigen required at the optimum zone was in one case (serum 10-USA-2a) approximately the same as with the homologous antigen; in the case of the other two sera, it

was far lower (one-thirty-sixth as much with serum 10-USA-4a). As with the 10-HSA sera, the precipitate with sera 10-USA-1a and 10-USA-2a was one-fourth to one-third as much as with homologous antigen. Only with the comparatively weak serum 10-USA-4a was the yield of precipitate with unmodified human serum albumin only slightly less than with the conjugated human serum albumin. However, the total yield of precipitate with this antigen was about the same for all three sera (0.33 milligrams, 0.33 milligrams, 0.38 milligrams.).

The reactions of the four 10-USA sera to demonstrate haptenic activity of 1,2-benzanthracene yielded only slight precipitates, even less than those with unmodified human serum albumin. The only precipitate greater than 0.26 milligrams was formed with serum 10-USA-1a and the antigen 10-Bov. All of the other precipitates demonstrating haptenic activity were less than those of the same type when sera against 10-HSA were used (see Table XXIII). For the one serum tested with 3,4-benzpyrene and 1,2,5,6-dibenzanthracene derivatives, the yield of precipitate was in all cases lower than with the 1,2-benzanthracene derivatives.

It is apparent from the results in Tables XXIII and XXIV that conjugation with the hydrocarbon caused considerable decrease of the antigenicity of the albumins. Sera produced by immunization with 10-HSA or 10-USA were capable of reacting with either part of the conjugated antigen used for injection. However, the quantitative studies showed that the 10-HSA conjugate was more effective in producing antibodies directed against the hydrocarbon group, since precipitates with heterologous conjugates were heavier with 10-HSA sera than with 10-USA sera.

DISCUSSION

The experimental results on the ability of these hydrocarbons to act as haptenic groups confirms the work of Creech and Franks (12, 21) with 1,2,5,6-dibenzanthracene. This compound showed a somewhat greater ability to act as a haptene in their work than in the experiments described above. Part of this difference may be due to the fact that Creech and Franks used only one other hydrocarbon, anthracene, in their comparative experiments; it is more probable that the main difference was caused by modifications in the experimental technique of preparing the conjugates. Landsteiner (41) had previously stated that anthracene, when coupled to a protein by an azo linkage, had only weak haptenic activity. In comparison, when anthracene and 1,2,5,6-dibenzanthracene were coupled to proteins by a carbamido linkage, Creech and Franks found that both hydrocarbons had rather marked serological specificities. Anthracene is not as satisfactory a haptene for comparison with 1,2,5,6-dibenzanthracene as are the compounds used in this study.

When the haptenic activity of 1,2,5,6-dibenzanthracene was compared with those of 1,2-benzanthracene and 3,4-benzpyrene, the experimental results indicated that the latter two hydrocarbons possessed much greater activity than 1,2,5,6-dibenzanthracene. The activity was not entirely dependent on the number of hydrocarbon groups attached to each albumin molecule of the immunizing antigen.

In the first series of experiments, the number of 1,2-benzanthracene groups, (i.e. thirty-eight) was almost twice that of each of the other hydrocarbons; this may have accounted in part for the higher

haptene activity of 1,2-benzanthracene. On the other hand, in the second series, the number of 1,2-benzanthracene groups in the horse serum albumin molecule was reduced to sixteen, yet the antigenic activity of this conjugate was still strong. The precipitation reactions with homologous and heterologous antigens of sera against the 1,2-benzanthryl-10-carbamido-horse serum albumin with sixteen groups were heavier than those of sera against 3,4-benzpyrenyl-5-carbamido-horse serum albumin with four groups, or 1,2,5,6-dibenzanthryl-9-carbamido-horse serum albumin with twenty-one groups. Therefore, even with approximately equal numbers of hydrocarbon groups in two of the conjugated antigens, there were significant differences in the activity of the hydrocarbons.

The number of hydrocarbon groups on the test antigens was also apparently not the factor determining the amount of precipitate. A serum against 1,2-benzanthryl-10-carbamido-human serum albumin was tested with bovine serum albumin derivatives of the three hydrocarbons, with 16, 15, and 19 hydrocarbon groups, respectively. The heaviest precipitate was formed with the homologous hydrocarbon conjugate, and lighter precipitates with the heterologous hydrocarbon conjugates; although the number of groups on each test antigen was approximately the same.

The linkage of isocyanates with proteins was considered by Hopkins and Wormall (27, 28, 29) to be with the free epsilon-amino groups of proteins, with the formation of a connecting carbamido group. This theory was supported by their experiments which showed that lysine derivatives of the haptene groups completely inhibited precipitation

reactions. Lysine is an amino acid with two amino groups, only one of which (the alpha amino group) is involved in peptide linkage within the protein structure. The other amino group, at the epsilon carbon atom, is not bound in peptide linkage, and would therefore be free to react with isocyanate groups. Inhibition of precipitation reactions by lysine derivatives of haptenes therefore indicated that the linkage was at the free epsilon amino groups, and that the protein structure immediately adjacent to the haptene group was also involved in their immunological activity.

Similar results were obtained in the experimental studies described in this thesis. Here, amino acid derivatives of 1,2-benzanthracene were used as inhibitors. The epsilon-amino caproic acid derivative of 1,2-benzanthracene caused marked inhibition of precipitation reactions due to haptene activity of 1,2-benzanthracene. Epsilon-amino caproic acid is different from lysine only in its lack of an alpha amino group. This is the amino group which is bound in peptide linkage when lysine is a structural unit of protein, where it would not be free to influence the immunological activity of the protein or protein conjugates. Therefore, since epsilon (1,2-benzanthryl-10-carbamido) -caproic acid strongly inhibited the precipitation reactions due to haptene activity of the hydrocarbon, it seemed most likely that linkage with protein was through the epsilon amino groups. The fact that other amino acid derivatives of 1,2-benzanthracene also inhibited the precipitation reactions does not detract from the major importance of this particular linkage. It is possible that under certain conditions the hydrocarbon isocyanates might combine with groups on the proteins other than the epsilon amino

groups. The work of Creech and Jones (15) with zein demonstrated that under the normal experimental conditions of conjugation, only free amino groups of the proteins were involved. The strong inhibition by the epsilon-amino caproic acid derivative, in contrast with the weak or moderate inhibition by other derivatives, supports the conclusion that the majority, if not all, of the linkages are of the carbamido type involving the free epsilon amino groups.

The quantitative studies of the precipitation reactions were of great value for accurate analysis of antigenicity. Although visual observation of precipitation reactions is of primary importance in determining the existence of precipitates, there are a number of factors which affect the value of this method for comparative studies.

When reading the literature on serological reactions which has accumulated during the last fifty years, one finds most often that the strength of precipitation was recorded as multiples of "+", i.e. "+", "++", "+++", etc. However, the quantity of serum and the concentration of antigen giving a specific reaction varied with each experimental study. Since the amount of precipitate is directly dependent on the concentration of both of these substances, only those reactions with the same concentration of components can be compared with any degree of accuracy. In some cases, the authors neglected to state the concentration of either reagent.

Another factor important in the visual method is the individual difference in recording the results. One observer may assign a value of "++" to the precipitate, while another person observing the same precipitate may assign to it only a "+" designation. There are no definite, absolute standards which can be used in visual observations

of precipitates; the accuracy of the method depends on the experience and reliability of the individual worker.

On the other hand, the protein content of the precipitate at the optimum zone is not influenced by the personal factor, once the technique of Kjeldahl analysis is mastered. If all the results are calculated on the basis of one cc. of antiserum standards of comparison then become available. Both the amount of antigen required and the amount of precipitate for reactions with homologous and heterologous antigens can be compared, and the degree of reactivity of the serum with various antigens can be more accurately determined. When this quantitative method comes into more general use in serological research, it may be possible that a graduated scale of values willbe established. Under such a system, the amount of precipitate resulting from any particular antigen-antibody reaction would fall in a definite place on the scale. The serologist would know with certainty whether he was studying a weak or a strong precipitation reaction. Then, too, the haptene activity of any compound could be measured in terms of a quantitative standard.

The carcinogenic hydrocarbons which have been studied in this research project definitely exhibit haptene activity, as measured by both visual observation and quantitative analysis of precipitation reactions. The three hydrocarbons could be evaluated by these methods on the basis of their effectiveness as haptenes in the following order of increasing activity: 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, and 1,2-benzanthracene. Whether or not one considers 1,2-benzanthracene a "strong" haptene group, in the light of the experimental evidence one

is led to the conclusion that it definitely does exert such determinant group activity, and that the immunological response is sufficiently non-specific to include reactivity with other hydrocarbons of related chemical structure.

The haptene activity of these hydrocarbons is provocative from the point of view of several of the current theories on the mechanism of antibody formation. Breinl and Horowitz (5) suggested that the orientation of amino acids in the antibody globulin molecule was governed by the surface residual valences of the antigen. They cited observations from Landsteiner's many experiments showing that antigens containing residual valency groups, i.e. carboxyl, amino, etc., incited antibody formation specific for the haptene group, whereas aliphatic or aromatic hydrocarbons, which lack such groups, were not capable of causing the formation of antibodies specific for the hydrocarbon group.

Mudd (47) suggested that the specificity of antibodies was a result of the arrangement of chemical groupings of the antibody molecule during synthesis, and that these groupings were adapted spatially and in their chemical affinities to the surface of the antigen.

Pauling's concept of antibody formation (49) was based on a bivalent antibody, i.e. a globulin molecule which had an immunologically active region at either end of the peptide chain. The particular configuration of the chain ends would be dependent, according to this theory, on the surface groups of the antigen, which by their charges attract or repel parts of the globulin chain. According to Pauling, the antibody molecule contains the same amino acids in the same relative order as an ordinary molecule (globulin) and differs from it only in the way in which the chain is coiled in the molecule.

In his book on serological reactions, Landsteiner (36) noted the results obtained by Creech and Franks (12, 15, 21) on the antigenicity of anthracene and dibenzanthracene. He stated:

> Quite possibly the hydrocarbon residues are responsible for the specificity of the reactions only in conjunction with adjacent parts of the protein.

The experimental evidence offered in this thesis does not contradict this statement. The marked inhibition of precipitation reactions by the epsilon-amino caproic acid derivative indeed appears to indicate that the adjacent part of the protein may be involved in the immunological specificity. Yet the hydrocarbon groups themselves must in some way have influenced the immunological response, because the results indicated that precipitation with homologous hydrocarbon-conjugate was in most cases greater than with heterologous hydrocarbon-conjugate.

Since it was most likely that all three hydrocarbons were conjugated with the same, or similar, structural groups on the serum albumins, the adjacent parts of the protein would have been alike. Therefore, the decrease in reactivity of antisera with heterologous hydrocarbons appeared to have been due primarily to a variation in structure from that of the homologous antigen. The chemical structure of each hydrocarbon was most probably responsible for the particular immunological response, as represented by antisera directed toward the hydrocarbon determinant group.

The chemical structure of the carcinogenic hydrocarbons is devoid of any functional polar groups, such as carboxyl, amino, nitro, etc. Landsteiner (34) and Landsteiner and Lampl (39) had shown, by their work on azoproteins, that introduction of only weakly polar groups, such as methyl or bromine, in the benzoic acid determinant group was of slight

influence on the immunological specificity of the conjugated antigen. Strongly polar groups, such as nitro groups, had greater influence on specificity.

It is probable that serological cross reactions among the carcinogenic hydrocarbons were the result of the lack of such groups in the molecular structure. It does not appear, however, that such groups are required in the structure of the antigen for the formation of antibodies directed against the haptene groups.

Since these hydrocarbons have been found to possess haptene activity, it is of interest to continue studies to determine if the immunological response extends to protection against their carcinogenic activity, and further, if such protection is effective against other hydrocarbons than the one used for immunization. The role of carcinogenic hydrocarbons in spontaneous tumor formation might be illuminated by a study of their immunological effect on the genesis of spontaneous tumors.

SUMMARY

1. The coupling of 1,2-benzanthracene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene with serum albumins by the carbamido linkage produced conjugated proteins possessing antigenic activity.

2. The conjugated proteins possessed a new specificity which was directed toward the hydrocarbon groups.

3. There was a moderate degree of serological cross reaction among the hydrocarbons. In most cases, however, the amount of precipitate with homologous hydrocarbon was greater than that with heterologous hydrocarbon.

4. Of the three hydrocarbons, 1,2-benzanthracene possessed the greatest haptenic activity, as measured both by visual observation and by the quantitative analysis of precipitates.

5. The alteration of the serological properties of the albumin portion of the conjugates was dependent on the hydrocarbon introduced and on the ease of the coupling reactions.

6. The crystalline horse serum albumin isolated in this laboratory appeared to have greater antigenic activity than the purified human serum albumin obtained from Dr. Edwin J. Cohn, of Harvard University. The 1,2-benzanthryl-10-carbamido derivative of horse serum albumin was the most effective produce in inciting the formation of antibodies against the haptene hydrocarbon group.

7. Quantitative micro-Kjeldahl analysis of precipitates formed at the optimum ratio of antigen and antibody served as an accurate method for comparisons of the haptenic activities of the hydrocarbons.

8. The precipitation reactions due to haptene activity of 1,2-benzanthracene were more strongly inhibited by the 1,2-benzanthryl deriva-

tive of epsilon-amino caproic acid than with other amino acid derivatives. This inhibition indicated that the hydrocarbon groups were linked to the serum albumins by a carbamido linkage with the free epsilon amino groups on the protein molecules.

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ACKNOWLEDGEMENTS

The author wishes to express her deep appreciation of the valuable aid of Dr. O. N. Allen in supervision of the research studies and in preparation of the thesis. The author is indebted to Dr. Hugh J. Creech for his encouragement and advice during the course of the research and thesis preparation, and for his preparation of most of the antigens used in this study. Many thanks are due to Messrs. Max Tryon and Edward Walton for some of the microKjeldahl analyses; to Mr. Larry Green for preparation of amino acid-hydrocarbon derivatives; and to Dr. Edwin J. Cohn for supplying the human serum albumin preparation.

Grateful acknowledgement is made to the International Cancer Research Foundation for the grant which made this work possible.

Evelyn Lenore Oginsky, Ph. D. 1943
(B. A. Cornell University
M. S. University of Chicago)

Title of thesis: An Immunological Study Involving Carcinogenic Hydrocarbons
Thesis directed by Professor O. N. Allen
Major: Bacteriology
Minors: Chemistry, Zoology
Pages in thesis, 92; Words in abstract, 353.

The purposes of this study were (1) to determine whether carcinogenic hydrocarbons act as haptenes when conjugated with proteins, and (2) to ascertain whether the immune sera are specific for the homologous hydrocarbon.

Horse serum albumin coupled by carbamido linkage with 1,2-benzanthracene, 1,2,5,6-dibenzanthracene, and 3,4-benzpyrene, and human serum albumin coupled with 1,2-benzanthracene were used as antigens. Rabbits were immunized by multiple intravenous and intraperitoneal injections totaling approximately 450 mg. of the conjugated or control native protein. Precipitin reactions with the resulting antisera were read macroscopically, and were confirmed quantitatively by microKjeldahl analyses of the washed precipitates.

The results indicate that the conjugated proteins had gained new specificity due to the determinant group. 1,2-Benzanthracene was found to be more effective as a haptene than the other two hydrocarbons. Antisera showing the best results were those formed by injection of 1,2-benzanthryl-10-carbamido-horse serum albumin. Although these sera reacted most strongly with the antigen used for injection, they demonstrated haptenic activity by reacting, to a lesser degree, with 1,2-benzanthracene conjugates from human and bovine serum albumin, and with

1,2,5,6-dibenzanthracene and 3,4-benzpyrene conjugates from bovine serum albumin. Quantitative analyses confirmed the observation that such cross-reactions were greatest at a definite ratio of antigen and antiserum; this ratio varied for the individual sera.

The use of amino acid derivatives of 1,2-benzanthracene as inhibitors of the precipitin reactions indicated that the epsilon-amino caproic acid derivative was more effective than those of glycyl-glycine, glycyl-glycyl-glycine, and glutamic acid. This finding is in agreement with evidence obtained previously by chemical means and indicates that this carbamido linkage with the hydrocarbon groups involves only the epsilon-amino groups of the serum albumins.

The immunological activity of the albumin portion of the conjugates was decreased by introduction of the hydrocarbon groups. Horse serum albumin had greater antigenic activity than human serum albumin, both as native protein and as the protein moiety of the conjugates.

The haptenic activity of these compounds makes desirable further research on protective immunization of animals against carcinogenesis due to various hydrocarbons.

VITA

Name in full: Evelyn Lenore Oginsky

Permanent address: Loch Sheldrake Road, Liberty, New York

Degree and date: Doctor of Philosophy; 1946

Date of birth: April 6, 1919

Place of birth: New York, New York

Secondary Education: Liberty High School, Liberty, New York; June 1934

Collegiate Institutions attended	Dates	Degree	Date of Degree
Syracuse University	1934-1935		
Cornell University	1935-1938	B. A.	June, 1938
University of Chicago	1938-1939	M. S.	August, 1939

Publications: An Immunological Study Involving Carcinogenic Hydrocarbons
Evelyn L. Oginsky, [illegible] Allen, and Hugh J. Creech.
Journal of Bacteriology, May, 1946. (In press)

Positions held:

1. Bacteriologist and ass't head of laboratory
   Richmond Memorial Hospital, Staten Island, New York
   October, 1939-May, 1940.

2. Laboratory technician
   Sibley Memorial Hospital, Washington, D. C.
   December, 1940-September, 1941.

3. Chemical and statistical analysis of smokeless powder
   War Department--Ordnance Department
   September, 1941-August, 1942.

4. Graduate Assistant, Department of Bacteriology
   University of Maryland, College Park, Maryland
   September, 1942-June, 1943.

5. Instructor, Department of Bacteriology
   University of Maryland, College Park, Maryland
   July, 1943- (present position).

6. Research Assistant, International Cancer Research Foundation
   University of Maryland, College Park, Maryland
   October, 1942-March, 1943; May, 1945-April, 1946.

7. Research Fellowship, Donner Foundation, Cancer Research Division
   (prospective occupation)