Thus conversion of cholesterol-4-C^{14} to DHEA-C^{14} must have taken place in the testicular tissue.

Discussion. The present findings show that the dog testis is able to convert cholesterol to DHEA and to secrete this steroid into its venous blood. It is unlikely that the DHEA isolated represents testicular metabolism of androstenedione or testosterone, since when these steroids labeled with C^{14} in position 4 were infused by the spermatic artery, no DHEA-C^{14} could be detected in spermatic vein blood.† Furthermore, administration of radioactive testosterone to human subjects will not result in excretion of radioactive DHEA in the urine(10).

The data of the present investigation, therefore, together with previous observations† (3), strongly suggest that DHEA is both produced and secreted by dog testicular tissue. Since the conversion of cholesterol to DHEA was lower than to testosterone or even addrostenedione(2) in all animals, it is at present difficult to assign biological importance to testicular secretion of DHEA in the dog. Furthermore, DHEA possesses only *ca.* 1/20 of the androgenicity of testosterone. It is possible that during synthesis of testosterone, where DHEA can serve as an intermediate† (3,4,11), excess DHEA may simply leak out from the interstitial cell and escape by the spermatic vein blood.

Summary. Dehydroepiandrosterone-C^{14} has been isolated in spermatic vein blood of anesthetized dogs infused with cholesterol-4-C^{14} by the spermatic artery. It is considered that this finding lends additional support to the view that dehydroepiandrosterone is an intermediate in the biosynthesis of testicular androgens.

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Properties of Serum Fractions Derived from Wheal Reactive Diphtheria Antitoxin.*† (27768)

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Allergic reagins or skin sensitizing antibodies possess the unique property of inducing wheal reactions following combination in skin with the corresponding antigen. This property may be recovered in serum fractions, although its distribution in these fractions is different from that of non-sensitizing antibodies. For example, skin sensitizing antibodies migrate electrophoretically as γ_1 globulins in contrast to non-sensitizing precipitating antibodies which migrate as γ_2 globulins (1,2,3,4). It is unfortunate that attempts to utilize such methods as zone electrophoresis,

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exchange resin chromatography and zone ultracentrifugation are attended by large losses in reaginic activity which accompany increased resolution of individual serum components. Kabat(5) has expressed the viewpoint that such fractionation may separate antibodies with different specificities and reactivities into different serum fractions. The basis for this reasoning is that the purified antigens used for immunization are frequently chemically heterogeneous and are thus potentially able to cause the formation of various sorts of antibodies directed against major and minor antigenic components. It is conceivable that some of these antibodies possess unusual electrophoretic properties as a matter of coincidence. However, this point of view has not provided an adequate explanation for the marked lability of allergic reagins as compared with ordinary antibodies, and there is general agreement that the reactivity of reagins as measured in skin tests differs sharply from other known forms of antibody(6,7). These observations have raised the possibility that increases in the purity of reagins result in separation of a serum protective factor from a protein with antibody-like activity. Stanworth(4) has suggested that a serum glycoprotein might fulfill this role.

We have found that when diphtheria antitoxic serum with wheal reactivity (previously termed skin sensitizing antitoxin or antitoxic reagin) was fractionated, considerable losses in skin activity occurred along with increased purification of individual serum fractions. However, it was of interest that fractions which contained most of the wheal reactivity contained mixtures of globulins, primarily gamma and α_2 globulins. Attempts to separate these components by physicochemical methods were attended by a marked diminution in wheal reactivity, particularly in components relatively free of α_2 globulin(8). These findings would support the idea of Stanworth(4) that a non-gamma globulin component plays a role in the biological reactivity of reagins. Details of these experiments are given here.

Two methods of cold ethanol fractionation were used to obtain components which were further subfractionated using the method of starch electrophoresis. Comparisons of skin sensitizing activity of fractions and subfractions were then carried out, and the titers obtained were expressed in terms of protein and antitoxin recoveries. The use of sensitive technics to titer antitoxin independently of protein and passive transfer tests enabled us to provide a valuable measurement of antibody activity. Losses of wheal reactivity in the various serum fractions and subfractions could also be calibrated in comparison with antitoxic activity as measured by the conventional methods(9).

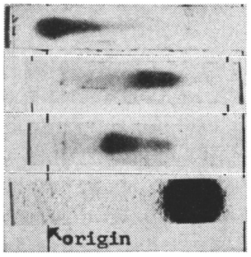
Materials and methods. Serum. Diphtheria antitoxic serum from one person was used. This individual (Hu) developed marked immediate wheal reactivity to intradermal purified toxoid following a single booster dose of purified diphtheria toxoid (10). The serum used in the present studies was obtained one month following booster toxoid; it contained 40 units per cc of antitoxin of the non-precipitating variety as determined by rabbit skin test. On the basis of this titer, as little as 0-025 unit was able to sensitize passive transfer sites to wheal reactions upon intradermal challenge with toxoid.

Assays. Antitoxin. Diphtheria antitoxin was assayed according to the rabbit neutralization test described by Fraser(9). Tests for precipitins in a semi-solid medium in the zone of excess toxoid did not show any precipitating bands in the one-month specimen, but a later specimen taken from this person showed a precipitating band which was not related to the toxin-antitoxin system(11).

Passive transfer. Whole serum or serum fractions were sterilized by filtration through Seitz filter pads. 0.1 cc portions were then injected intradermally in duplicate sites on the back or arms of normal Schick positive recipients who were themselves not sensitive to diphtheria toxoid. Challenge was at 24 hours using 0.1 Lf. of the immunizing toxoid (equivalent to 0.005 μg N) contained in 0.05 cc. Wheal and erythema reactions were measured 15 minutes after challenge(12). "Wheal reactive dose" is defined as the minimum amount of protein or antitoxin which causes an immediate reaction of 1+ or greater, 15-

TABLE I. Properties of Skin Sensitizing Antitoxin Serum Fractions Obtained by Method 6 of Cohn *et al.**

Designation of fraction	Wt of fraction (g)	Antitoxic units (rabbit skin test)	Antitoxic units per g protein	Antitoxin units required to elicit wheal reaction	Electrophoretic patterns of fractions — +
II + III†	1.730	3.2×10^3	1.9×10^3	.025	
IV-1	.275	5×10^1	1.8×10^2	>.1	
IV-4	.235	1×10^1	4×10^1	>.1	
V	2.110	0		—	
Totals	4.350 75% of original	3.26×10^3 *ca.* 80% of original			



* Initial volume of serum—100 cc; protein content—6 g; antitoxin content—4×10^3 units.
† Physical conditions—pH 6.6, μ 0.1.

30 minutes after challenge with antigen.

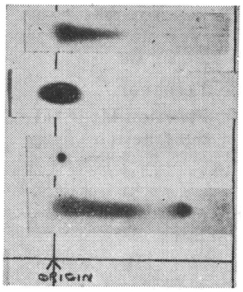
Methods of serum fractionation. Cold ethanol precipitation. Two systems of cold ethanol precipitation were employed(13,14). In both methods, fractions differing in globulin or albumin content were obtained by controlling 5 different variables: (1) ethanol concentration, (2) temperature, (3) ionic strength, (4) hydrogen ion concentration, and (5) protein concentration. In the first of these methods (Cohn Method 6) 5 fractions designated I, II + III, IV + 1, IV-4 and V are ordinarily separated from plasma, but in the present experiments fraction I was not obtained since serum was used as starting material. Fraction II + III contains 70-80% of the γ globulins of plasma. In the second method, 2 fractions designated γ_1 and γ_2 globulins have been separated which differ widely in isoelectric point (pH 5.7 and 7.3, respectively, in 0.1 ionic strength buffers) and electrophoretic mobility. The second fractionation technic was employed to separate the components in fraction II + III obtained by Cohn Method 6.

Starch electrophoresis. Separation of serum fractions by starch electrophoresis was carried out according to a method described by Kunkel(15). Following electrophoresis, one-half inch segments of starch were cut, and the proteins eluted using 2 cc of normal saline. Protein, antitoxin, and passive transfer studies were carried out on eluates which had been filtered as described above. Protein analyses were obtained using a modified Folin-Ciocalteau procedure(16); values for optical density were converted to milligrams of protein employing a suitable standard curve (17), and total recoveries of protein were estimated by multiplying the converted figure times volume of each eluate. Estimates of total antitoxin were likewise based upon the number of units of antitoxin/cc times volume of eluate.

Results. Table I describes the properties of fractions II + III, IV-1, IV-4 and V derived from whole serum Hu. Seventy-five per cent of the starting protein content was recovered in these components. Eighty per cent of the original antitoxin was recovered and was contained largely in fraction II + III, a protein comprising 28% of the original starting material. Fraction II + III retained the same amount of wheal reactivity proportionate to antitoxin content as compared with activity present in whole serum. Wheal reactivity was small or non-existent in fractions IV-1, IV-4 and V. Table II indicates the yields and biological properties of subfractions derived from Cohn fraction II + III using the method of Deutsch *et al.*(14). Fifty-nine per cent of the starting protein and 63% of the antitoxin was recovered, distributed in 4 fractions designated γ_1, γ_2, β, and

TABLE II. Properties of Cold Ethanol Fractions Derived from Cohn Fraction II + III Antitoxic Globulin. Technic of Deutsch *et al.*

Designation of fraction	Physical condition needed to obtain globulin	Wt of fraction (g)	Antitoxic units (rabbit skin test)	Antitoxic units per g protein	Antitoxin units required to elicit wheal reaction	Electrophoretic patterns of fractions — +
γ_1 globulin	μ .05-.1	.138	3×10^2	2.2×10^3	.025	
γ_2 globulin	μ .005	.329	1.2×10^3	3.6×10^3	.5	
β globulin		.277	1×10^2	3.6×10^2	>.1	
α globulin	μ .05-.1	.277	4×10^2	1.4×10^2	.025	
Totals		1.021 59% of original	*ca.* 63% of original			



crude α. Fractions γ_2 and crude α globulin dissolved readily upon admixture with water. Fractions γ_1 and β, particularly the latter, dissolved poorly when in mixture with water. Sixty per cent of the recovered antitoxin was present in γ_2 globulin; the remainder was distributed in other subfractions. The passive transfer properties of fractions and subfractions compared with whole serum is indicated in Table III. All of the designated fractions (II + III, γ_1, and crude α) except γ_2 behaved similarly in passive transfer in relation to their antitoxin content. Although relatively inactive in passive transfer tests, γ_2 globulin contained antitoxin concentrated 5 to 6 times above its concentration in whole serum. On the other hand, the γ_1 and crude α subfractions retained wheal reactivity proportionate to antitoxin content in the presence of a marked reduction in protein as compared with the original fraction. Thus, subfractions γ_1 and α behaved similarly to fraction II + III and whole serum in regard to their passive transfer properties in the presence of 7-fold and 20-fold reductions in total protein, respectively (Table IV).

The electrophoretic analysis of fraction II + III and of γ_1, γ_2 and α globulins derived from fraction II + III is presented in Fig. 1. Fraction II + III is largely distributed as a gamma and beta globulin, with additional small amounts of the other globulins and albumin. Total protein recovered was 13 mg, from an original starting protein content of 50 mg. A part of this loss was due to the presence of considerable insoluble residue following reconstitution of fraction II + III with distilled water. This residue was removed by centrifugation prior to electrophoresis. Removal of electrophoretic eluates which contained only gamma globulin provided a concentrated antitoxin prepa-

TABLE III. Passive Transfer of Sensitivity to Diphtheria Toxoid: Comparison of Antitoxic Whole Serum (Hu) with Fractions Obtained by Cold Ethanol Precipitation.

Sensitizing dose (units of antitoxin)	Name and potency of fraction				
	Whole serum 6 g% protein	II + III 1.73 g% protein	γ_2 globulin .329 g% protein	γ_1 globulin .277 g% protein	α globulin .277 g% protein
.1	++++	++++	+	++++	++++
.5	++++	++++	0	++++	++++
.25	++++	+++	0	+++±	++++
.05	+++	++±	0	+++	+++±
.025	++	+±	0	+±	++±
.01	±	0	0	tr	+
.005	0	0	0	0	0

TABLE IV. Antitoxic Activity and Immediate Wheal Reactivity in Fractions Derived from Diphtheria Wheal Reactive Antitoxic Serum (Hu).

Designation of component	Units of antitoxin per g of protein	Wheal reactive dose* expressed as Protein (mg)	Antitoxin (unit)
Whole serum	6.7×10^2	.06	.025
Cold ethanol			
Fraction II + III	1.9×10^3	.02	.025
Subfraction γ_1	2.2×10^3	.003	.025
" γ_2	3.6×10^3	.3	.5
" crude α	1.4×10^3	.003	.025
Starch electrophoresis eluates			
II + III (13-16)	3.3×10^4	.0025	.025
γ_1 (13)	1.0×10^5	>.003	>.3
γ_1 (14-15)	2.7×10^4	>.001	>.01
γ_1 (17-18)	6.2×10^3	.002	.01
γ_2 (10-13)	1.0×10^4	>.005	>1.
α (13)	3.3×10^5	>.003	>.1
α (14-16)	2.8×10^4	>.0025	>.1
α (17-20)	9.7×10^3	.003	.01
α (21-22)	7.5×10^2	.004	.01

* Measured by method of passive transfer.

ration with little impairment of wheal reactivity as demonstrated in passive transfer tests (Table IV).

γ_2 globulin was derived in these experiments as an almost pure electrophoretic subfraction. The recovery of protein from starch was almost 100% of the original starting quantity (39 mg of 40 mg). This material contained little or no demonstrable β or α globulin. Its wheal reactivity as compared to antitoxin and protein content was comparatively small (Tables III and IV). On the other hand, the electrophoretic mobilities of the γ_1 and crude α globulins were heterogeneous. The recovery of γ_1 globulin protein was 57% (13 of 23 mg) and of crude α globulin, 82.5% (33 of 40 mg) of the original starting protein content. The distribution of wheal reactivity in both fractions was proportionate to antitoxin content in the electrophoretic areas which migrated as β or α globulins. These were the eluates from segments numbered 17 and 18 in the γ_1 globulin, and eluates from segments 17 to 22 in crude α globulin. However, protein recovered from the areas which contained most of the antitoxin (*i.e.*, segment 13 eluates for both γ_1 and α globulin) was far less active in passive transfer as compared with the activity of these globulins prior to electrophoresis (Table IV). Table IV indicates that progressive concentrations of antitoxin within gamma globulin fractions occurred along with increasing resolution of gamma globulin by electrophoresis. On the other hand, wheal reactivity was greatly impaired in highly purified gamma globulin fractions containing antitoxin, except in antitoxic fragments which possessed non-gamma globulin components. No wheal reactivity was observed in fractions devoid of antitoxin or in fractions containing only albumin.

Discussion. Earlier experiments in our laboratories have indicated that electrophoretic fractions derived from antitoxic serum possessing wheal reactivity retain much of the original skin sensitizing activity (1,18). According to the present studies, this also seems to be true of fractions which are heterogeneous in their globulin content and which include gamma globulin. The electrophoresis experiments carried out on γ_1 globu-

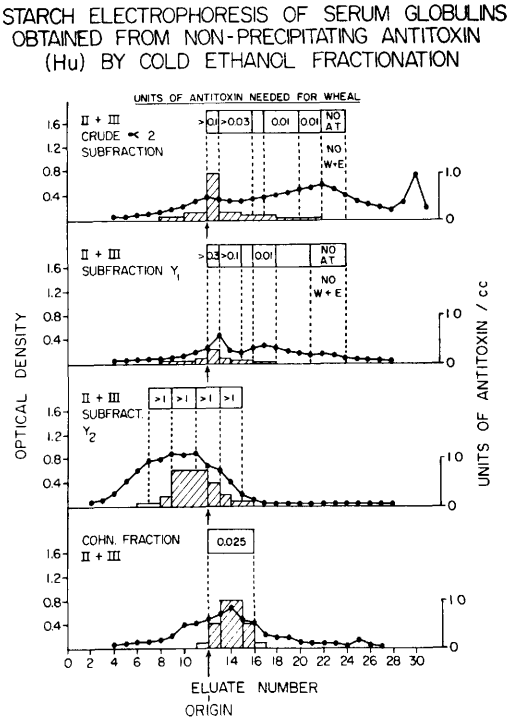


FIG. 1

lins obtained by cold alcohol precipitation indicate that wheal reactivity is best expressed when a fast moving gamma globulin and a non-gamma globulin component (β or a_2 globulin) coexist. However, skin sensitizing activity is markedly attenuated in highly purified γ_2 globulin preparations and in albumin. The labile quality of the skin sensitizing property in purified serum fractions has been commented upon by other investigators(3,4,6).

The possibility that antitoxin is measured separately from an antibody which reacts only in skin has been considered(19). This seems unlikely on the basis of other characteristics of serum Hu which have been described elsewhere(10). However, Finger and Kabat(11) raise the possibility based upon the existence (in a sample taken 3 years later) of a precipitating system unrelated to toxin and antitoxin.

The following findings suggest a selective removal from serum of a protective factor for reagins. Progressive resolution and concentration of serum fractions was not associated with increases in wheal reactivity and, therefore, none of the fractions tested were appreciably stronger in their degree of wheal reactivity than was whole serum itself. On the other hand, relatively high concentrations of antitoxin could be attained when subfractionation of gamma globulin fractions was carried out, although the wheal reactivity of such fractions invariably diminished as resolution of gamma globulin became more distinct. Thus antitoxic reagin may be comprised of antibody modified by the coexistence of another labile protein to account for its biological and other extraordinary properties(18). It is of pertinence in this regard that heating of serum Hu at 56°C caused a loss of the wheal property with a conversion to blocking antibody as demonstrated in passive transfer tests(10).

Whether the present methods are optimal for isolation and concentration of antitoxic reagin is not known. The over-all loss in protein in some of the fractionation procedures was considerable, and this may have a bearing upon our interpretation of the present evidence. An alternate viewpoint which cannot be answered by the present work is that reagins were inadvertently lost in portions of serum not recoverable under the experimental conditions. It is of interest that Heremans(20) has recently described a fractionation method not related to the present technics which yields concentrated wheal reactive subfractions.

Regardless of the point of view concerning the exact chemical nature of reagins, there is sufficient evidence concerning their *in vivo* and *in vitro* behavior to indicate that they are very unusual in comparison to so called ordinary antibodies. This is of great importance in considering the possible heritable nature of atopy(21). If it is asumed that reagins signal atopy as either a past, present or future condition, it is also reasonable to assume that humoral and cellular conditions are altered from the normal to permit the development of reagins. It is important to determine whether such circumstances are truly heritable or whether normal cell lines in immunity are peculiarly susceptible to certain environmental situations. One such situation would be the continued or frequent existence of antigen-antibody complexes, an event which would be fostered by the frequent repeated administration of soluble protein antigens (19). In keeping with the idea that complexes play a crucial role, another possible inherited factor to be ruled out is a metabolic defect which might favor the prolonged existence of complexes once formed.

Summary. Properties of fractions of diphtheria antitoxic wheal reactive serum have been described. The ability of these fractions to sensitize human skin has been related to their protein and antitoxin content. Two methods of cold ethanol fractionation were employed to obtain serum components, some of which were then subfractionated electrophoretically in a starch supporting medium. The results of passive transfer tests suggest that skin sensitization is best expressed in fractions which contain gamma globulin and a non-gamma (β or a_2) globulin. The point of view is adopted that the serum under consideration contains antitoxic reagins, and that they represent antitoxin whose behavior is modified by the coexistence of a labile protein protective serum factor.

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Thyroid Compensation Under the Influence of Dietary Nitrate.* (27769)

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(Introduced by B. L. O'Dell)
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The ability of thiocyanate, perchlorate, nitrate and other anions to interfere with normal thyroid function has been the basis of numerous investigations(1,2). Of these ions, nitrate is unique in that it is found in many plant species(3) and in well water(4,5) commonly consumed by domestic animals and man. Recent studies(6) have shown that dietary nitrate interferes with the normal metabolism of injected I^{131}. These studies were conducted on rats and sheep fed nitrate for less than one week. Under normal conditions rats fed KNO_3 show a slight depression in rate of gain; however, when cold stress was applied, the rate of gain was significantly decreased.† This would indicate that rats can compensate for a nitrate load under the usual environmental conditions but not under cold stress. This investigation was undertaken to determine whether or not the thyroid compensates for the nitrate effect on iodine metabolism.

Materials and methods. Sixteen 150 g male rats, originally of the Wistar strain, were divided into 2 groups of 8 each. The animals were maintained on a basal diet of the following % composition: ground yellow corn 58.5, soybean oil meal 35.0, calcium carbonate 1.5, dicalcium phosphate 1.5, sodium chloride 0.2, and Vit. B mix 0.8 (diet calculated to contain 0.83% K and 0.17% Cl). The animals were fasted 24 hours then fed the basal diet supplemented with 2.5% KNO_3 or 1.8% KCl plus 0.7% cerelose. Six hours after the diets were offered, the animals were injected intraperitoneally with 400 μg tapazole per 100 g body weight. One hour later

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