THE REQUIREMENT FOR HYDROCORTISONE IN ANTIBODY-FORMING TISSUE CULTIVATED IN SERUM-FREE MEDIUM*

By CHARLES T. AMBROSE, M.D.

(From the Department of Bacteriology and Immunology, Harvard Medical School, Boston)

(Received for publication, February 20, 1964)

The investigations reported here stem from the discovery that the secondary response can readily be elicited in cultures of lymph node fragments prepared from previously immunized rabbits (1). Like most other organ and tissue culture systems, these lymph node cultures were originally found to require the presence of serum in a medium otherwise chemically defined in its content of salts, glucose, amino acids, and vitamins. But the secondary response was noted to vary in media containing sera from different rabbits, sera from different bleedings of the same rabbit, or even samples of the same serum stored frozen for different periods. Thus the presence of serum is obviously undesirable because of its variable and poorly defined composition. Recently, we found that equally good antibody responses were obtained with culture medium in which serum was replaced by physiological levels of hydrocortisone (2). The present report describes in detail the experiments leading to this observation and correlates it with (*a*) related nutritional findings in other tissue cultures, (*b*) various effects of corticosteroids on other culture systems, and (*c*) the influences of these hormones on the immune response both *in vitro* and *in vivo*.

Materials and Methods

The general procedures employed in these experiments have been described by Michaelides and Coons (1) and Ambrose and Coons (3); they are briefly as follows: healthy adult male albino rabbits received a primary stimulation *in vivo* to both bovine serum albumin (BSA) and diphtheria toxoid by injections in both hindfoot-pads and in both ears, and also in some rabbits in both front foot-pads. The total amount of antigen injected intracutaneously into all 4 or 6 sites varied from 24 to 60 mg for BSA and from 0.70 (240 Lf) to 2.4 mg (816 Lf) for diphtheria toxoid. Six to 20 weeks later the regional lymph nodes draining the injection sites were excised aseptically and cut into 1 mm cubes. The fragments received a secondary stimulation *in vitro* during a 2 hour incubation in medium containing BSA, 0.5 mg/ml, and diphtheria toxoid, 0.014 mg/ml (5 Lf/ml). Excess antigen was washed from the fragments by several rinses with medium. Twelve stimulated fragments were inserted into a Leighton tube and a thin pad of glass wool was laid over them. When moistened with medium, the glass wool pad clings to the inner surface of the tube and holds the underlying fragments in position. Generally, 3 to 4 such cul-

* This investigation was supported by Grant H-2255 from the United States Public Health Service.

ture tubes were prepared for each variable in an experiment. Each tube received 1 ml of Eagle's medium containing 25 per cent normal rabbit serum or various other supplements to be described. Sufficient media for an entire experiment were prepared, sterilized by filtration through Millipore membranes (0.45 μ pore size) and stored in screw-capped tubes in the refrigerator. The culture tubes were kept stationary at 37°C for 2 to 6 weeks; the culture fluid was replaced every 3 days. The fluid removed from each culture was immediately frozen in an alcohol–dry ice bath and stored at −5°C. Antibody titers to BSA and diphtheria toxoid were subsequently measured by the hemagglutination method with tanned sheep erythrocytes (Stavitsky's modification of the Boyden procedure, reference 4).

Mathematical Treatment of the Data.—The total antibody produced by a culture during a selected interval in each medium was compared with that produced in one of two reference media. In the early experiments described in this paper the reference medium was Eagle's

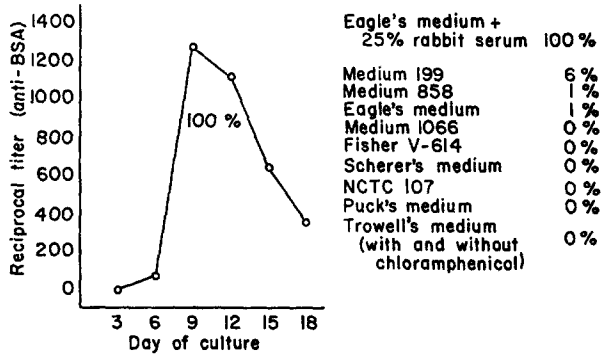


FIG. 1. A typical secondary antibody response in regular medium following antigenic stimulation on day 0 *in vitro*. Each point is the average of the reciprocal titers of four identically treated cultures. The cumulative 18-day responses of cultures in various chemically defined media are indicated to the left of the graph.

medium containing 25 per cent normal rabbit serum. In later experiments, the reference medium was a serum-free medium containing hydrocortisone.

Fig. 1 illustrates a typical secondary response of lymph node fragments. Culture fluids were removed every 3 days from four identically treated cultures; each point on the graph represents the average of the reciprocal hemagglutination titers of these four fluids. The total antibody produced by these cultures during their 18 day life was estimated by adding the reciprocals of the average titers of the six medium changes and was designated as a 100 per cent response. Comparison of this value with similarly derived values for fragments cultured in various other experimental media yielded the percentage response in each medium. For example, in the first experiment (Fig. 1) the total average antibody response of the four cultures maintained in the reference medium was 3470; the total response of the fragments cultured in Eagle's medium without serum was 35. Thus, the response in Eagle's medium was only 1 per cent of the response in the reference medium.

Limitations in Titration and Culture Techniques.—The 2-fold dilutions used in the hemagglutination titration provide, of course, a relatively crude value for the antibody content. However, errors in titration can generally be readily detected because of the regular shape of the response curve when the composition of the medium is held constant throughout the life of the culture.

Another weakness in the present experimental method is the variability of lymph node fragments in replicate culture tubes. Fragments vary in size despite care in their preparation; those from different nodes or from separate areas of the same node contain different numbers of antibody-producing cells. The effect of these variables on the total response can be reduced by the random selection of twelve fragments of equal size for each culture tube and the random assignment of the tubes to each group in the experiment. With these precautions, there is close agreement in most experiments in the titers of identically treated cultures.

RESULTS

The secondary response by lymph node fragments stimulated *in vitro* occurs in Eagle's medium only when serum is present.[1] Negative results with our culture system were obtained in all chemically defined (*i.e.* serum-free) media tested (Fig. 1), although most of them have supported the continuous growth of some variety of mammalian cells in cultures (5, 6). In addition to the 28 "essential" ingredients in Eagle's medium, these synthetic media contain other amino acids, vitamins, nucleic acids derivatives, sugars, coenzymes, lipids, and other substances (*cf.* Table VIII of Parker, reference 5). The failure of these additional substances (more than 50 in number) to support antibody production indicates that the component of serum essential for antibody production is not among them.

The Effect of Dialyzed Serum and Serum Dialysate.—

The amino acid and vitamin requirements of several mammalian cell lines were determined by Eagle (7) in experiments with media containing 5 per cent dialyzed serum. We dialyzed rabbit serum against running tap water at 5°C for 72 hours and then against several changes of normal saline-phosphate buffer (8) pH 7.0 at 5°C for 24 hours.

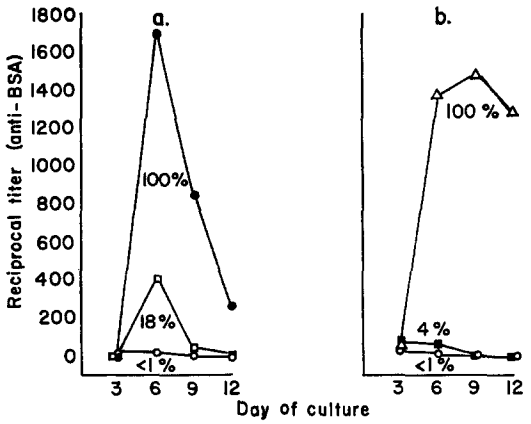
In several successive experiments the secondary response was found to be completely inhibited in Eagle's medium containing 25 per cent dialyzed serum but was good in medium containing undialyzed serum from the same serum pool. The results of one such experiment are shown in Fig. 2 *a*. In the same experiment, Fig. 2 *b*, the antibody response in Eagle's medium containing 12 per cent normal rabbit serum was equal to that in the reference medium. But the mixing of 25 per cent dialyzed serum with 12 per cent normal serum abolished the serum effect; here the response was only 4 per cent of that obtained in Eagle's medium supplemented with 12 per cent serum alone.

An explanation for this unexpected result was suggested by the following experiment. A solution was prepared from normal rabbit serum by dialyzing it against an equal volume of distilled water with gentle shaking at 5°C for 24 hours. In Eagle's medium containing 25 per cent dialyzed serum and 50 per cent of this dialysate, a significant response, 18 per cent, occurred (Fig. 2 *a*). In other experiments similarly prepared dialysates alone successfully substi-

[1] Except in stressed rabbits; this is discussed below.

tuted for serum. Figs. 3 *a* and 3 *b* depict the antibody responses in reference medium (100 per cent), in Eagle's medium alone (*i.e.* without serum) (11 per cent), in Eagle's medium plus 50 per cent dialysate (72 per cent), and in Eagle's medium containing 50 per cent of an 8-fold concentrated dialysate (110 per cent).

Thus the secondary response *in vitro* can be supported by the dialyzable components of serum and does not require serum proteins in the medium. The unexpected finding, described above, that 25 per cent dialyzed serum inhibits the



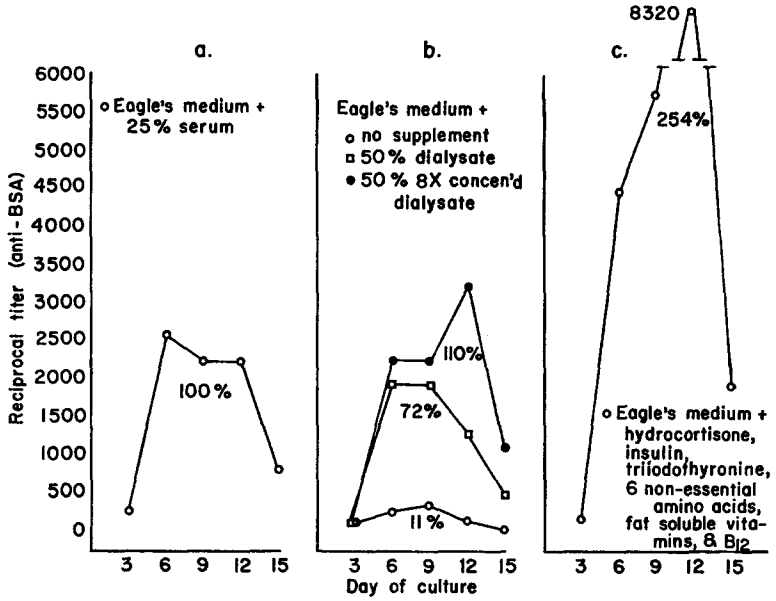
FIGS. 2 *a* and 2 *b*. The secondary antibody responses in Eagle's medium containing 25 per cent serum ●——●, 25 per cent dialyzed serum ○——○, 25 per cent dialyzed serum plus 50 per cent dialysate □——□, 12 per cent serum △——△, 12 per cent serum plus 25 per cent dialyzed serum ■——■.

antibody response in the presence of 12 per cent normal serum, suggests that the proteins in the exhaustively dialyzed serum tightly bind the dialyzable substance needed for the secondary response and provided by normal serum. These observations led us to try to identify the active substance in the dialysate.

The Effect of Hormones, "Non-Essential" Amino Acids, and Additional Vitamins.—

Because many hormones are present in serum in both a free and protein-bound state, we investigated the effect of some of them on antibody synthesis *in vitro*. The hormones initially studied were hydrocortisone (hydrocortisone sodium hemisuccinate) and triiodothyronine. At that time we were also interested in the possible effects of insulin, fat-soluble vitamins, vitamin B_{12}, and Eagle's non-essential amino acids,—those found to be unnecessary for the mammalian cell lines Eagle tested (7) and hence not present in his mixture of "essential" amino acids.

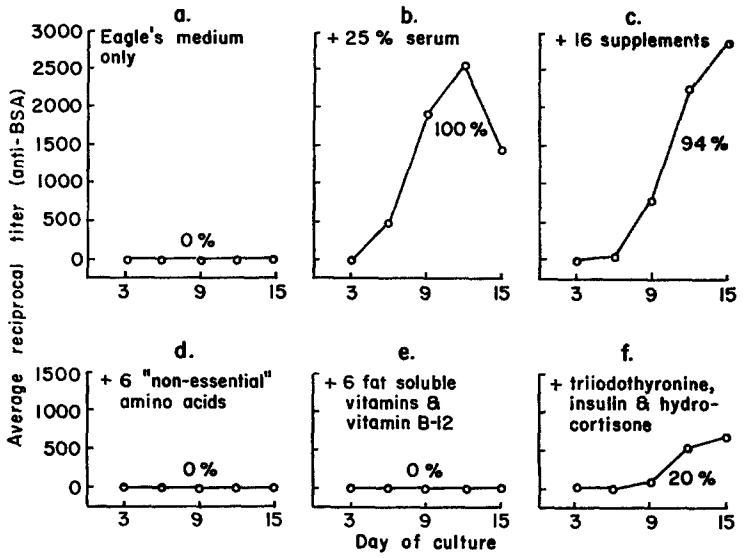
Addition of these 16 substances[2] to Eagle's medium *without serum* supported a response of 254 per cent (Fig. 3 *c*), far greater than that produced in the reference medium (100 per cent) or in Eagle's medium fortified with concentrated dialysate (110 per cent).



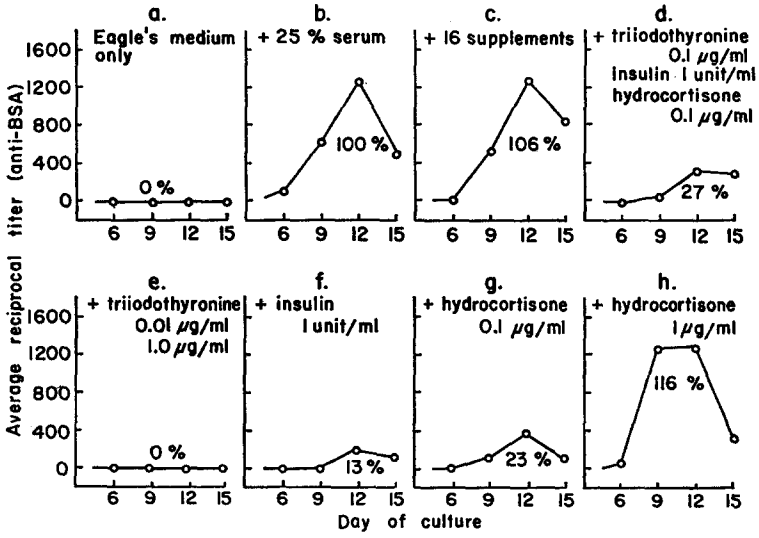
FIGS. 3 *a* to 3 *c*. The secondary antibody responses in Eagle's medium containing serum serum dialysates, or a mixture of 16 chemically defined supplements (*cf.* text and footnote 2)

The next two experiments (Figs. 4 and 5) revealed that hydrocortisone and, to a lesser degree, insulin were the active components in the mixture of 16 substances. Insulin (1 unit/ml) produced a 13 per cent response (Fig. 5 *f*); hydro-

[2] The final mixture of 16 components was as follows: 0.1 mM each of six "non-essential" amino acids (alanine, asparagine, aspartic acid, glycine, proline, and serine), 0.25 μg/ml vitamin A, 10 μg/ml vitamin E, 0.45 μg/ml vitamin K, 5 μg/ml vitamin K, 5 μg/ml linoleic acid, 5 μg/ml linolenic acid, 5×10^{-6} μg/ml thioctic acid, 2×10^{-4} μg/ml vitamin B_{12}, 0.01 μg/ml triiodothyronine (Smith, Kline and French Laboratories, Philadelphia, cytomel), 1.0 unit/ml crystalline zinc insulin (Eli Lilly and Co., Indianapolis, iletin), and 0.1 μg/ml hydrocortisone sodium hemisuccinate (Parke, Davis and Co., Detroit, solu-cortef). In several experiments the number of non-essential amino acids used was seven with the inclusion of 0.1 mM of glutamic acid. The fat soluble vitamins were dissolved in alcohol at 200 times the final concentration desired in the medium; thus alcohol comprised only 0.5 per cent of the final volume of the medium. In other experiments inhibition of antibody synthesis did not become evident until the alcohol content of the medium was greater than 1 per cent. The amino acids and vitamins employed were all obtained from California Corporation for Biochemical Research, Los Angeles.



FIGS. 4 *a* to 4 *f*. The secondary antibody responses in unsupplemented Eagle's medium (Fig. 4 *a*) and in Eagle's medium supplemented with serum (Fig. 4 *b*), the 16 supplements (Fig. 4 *c*), or groups of these supplements (Figs. 4 *d* to 4 *f*) (*cf.* text).



FIGS. 5 *a* to 5 *h*. The secondary antibody responses in unsupplemented Eagle's medium (Fig. 5 *a*) and in Eagle's medium supplemented with serum, (Fig. 5 *b*), the 16 supplements (Fig. 5 *c*), the 3 hormones (Fig. 5 *d*), or the individual hormones as indicated (Figs. 5 *e* to 5 *h*) (*cf.* text).

cortisone (1.0 μg/ml) produced a 116 per cent response (Fig. 5 *h*). Antibody production was not supported by Eagle's medium plus the 6 non-essential amino acids (Fig. 4 *d*), the extra fat-soluble vitamins and vitamin B_{12} (Fig. 4 *e*), or 0.01 to 1.0 μg/ml of triiodothyronine (Fig. 5 *e*). However, both experiments

TABLE I

Amino Acid Requirements

	Amino acid(s) omitted	Reference response
		per cent
	None (reference response)	100
	All 20	0
Eagle's 13 "essential" amino acids	Cystine	0
	Valine	0
	Tryptophane	0
	Threonine	1
	Isoleucine	2
	Lysine	2
	Methionine	2
	Arginine	5
	Histidine	10
	Tyrosine	18
	Phenylalanine	25
	Leucine	37
	Glutamine	115
7 "non-essential" amino acids	Serine	3
	Glycine	38
	Proline	49
	Asparagine	58
	Aspartic acid	67
	Alanine	100
	Glutamic acid	127

Cumulative anti-BSA responses over 18 days in media lacking individual amino acids. This experiment was performed in Eagle's medium (without serum) supplemented with 0.1 mM of each of the 7 non-essential amino acids, 0.5 units/ml insulin, and 0.1 μM hydrocortisone sodium hemisuccinate but lacking the amino acid(s) indicated.

suggested that either the non-essential amino acids or the extra vitamins augmented the effect of the two hormones. The responses in Eagle's medium with all 16 supplements (94 per cent in Fig. 4 *c* and 106 per cent in Fig. 5 *c*) were considerably greater than those obtained in Eagle's medium containing only the three hormones (20 per cent in Fig. 4 *f* and 27 per cent in Fig. 5 *d*), only insulin (13 per cent in Fig. 5 *f*), or only hydrocortisone (23 per cent in Fig. 5 *g*).

Non-Steroid Nutritional Requirements.—Since hydrocortisone effectively re-

places serum in this culture system, precise studies on the amino acid and vitamin requirements for the secondary response *in vitro* became practicable. Data of a preliminary nature are summarized in the first two tables. Table I lists the antibody responses in serum-free media deficient in a single amino acid. For these experiments the reference medium was Eagle's medium supplemented with the 7 non-essential amino acids, insulin, and hydrocortisone (see Table I).

The omission of either glutamine or glutamic acid did not impair the response, probably because each can substitute metabolically for the other at the concentrations employed here (9) The omission of leucine or phenylalanine reduced the response significantly; however, omission of any one of the other 10 essential amino acids produced even greater reduction. Of the non-essential amino acids the omission of serine most sharply depressed the antibody response. Omission of the other non-essential ones, like phenylalanine and leucine, reduced the response considerably less, suggesting that these amino acids may be partly supplied by the cells' own synthetic activity or by the cell lysis.

In a similar experiment individual vitamins were omitted from the mixture of eight present in Eagle's complete medium (Table II). The 100 per cent antibody response was that produced by cultures in Eagle's medium containing all eight vitamins as well as hydrocortisone, insulin, and vitamin B_{12}. The omission of folic acid, calcium pantothenate, inositol, or riboflavin reduced the total response to less than 25 per cent. The deletion of other vitamins had less effect, perhaps because intracellular reserves of these vitamins sufficed during the culture's 21 day life.

The contribution of insulin and vitamin B_{12} in the medium to antibody production has been difficult to confirm. In one experiment Eagle's medium supplemented with the non-essential amino acids and 0.1 μM of hydrocortisone produced a 21 day anti-BSA response which was taken as 100 per cent. The addition of 0.5 units/ml of insulin to this medium increased the response to 148 per cent; similarly, the addition of 0.5 μg/ml of vitamin B_{12} raised the response to 139 per cent. In other experiments, antibody production was not enhanced by these two substances. These substances may be present in the lymph nodes of healthy rabbits in amounts sufficient for the metabolic requirements of the secondary response initiated *in vitro*. A requirement for these substances in the medium might be revealed in fragments from vitamin- or insulin-deficient rabbits.

Metabolic Requirements for Corticosteroids.—Comparable data from eight experiments have been collected in Table III to show the effect of the hydrocortisone concentration on the secondary response *in vitro*. No appreciable response was supported by 0.001 μg/ml or less, while significant antibody production occurred in the presence of 0.01 μg/ml or more. In Table III the maximum responses for each experiment are set in boldface type and were produced in

medium containing 0.1 to 10 μg/ml of hydrocortisone (0.2 to 20 μM). However, in Experiments 103 and 113 the responses to 1.0 μg/ml were greater than those to 10.0 μg/ml. Therefore, 0.1 to 1.0 μg/ml (0.2 to 2.0 μM) is the optimal hydrocortisone concentration for the secondary response under the present culture conditions.

In most of our experiments little or no antibody was produced in Eagle's medium or in other serum-free media lacking corticosteroids; however, in two experiments listed in Table III significant amounts were produced in Eagle's medium compared to the maximum amounts formed in the hydrocortisone-

TABLE II

Vitamin Requirements

Vitamin(s) omitted	Reference response
	per cent
None (reference response)	100
All 8	0
Folic acid	5
Calcium pantothenate	9
Inositol	21
Riboflavin	24
Thiamin·HCl	39
Pyridoxal·HCl	57
Choline chloride	62
Nicotinamide	89

Cumulative anti-BSA responses over 21 days in media lacking individual vitamins. This experiment was performed in Eagle's medium (without serum) supplemented with 0.1 mM of each of 6 non-essential amino acids (asparagine was omitted), 0.5 μg/ml vitamin B_{12}, 0.5 unit/ml insulin, and 0.1 μM hydrocortisone sodium hemisuccinate but lacking the vitamin(s) indicated.

supplemented medium, 7 per cent and 39 per cent in Experiment 109 and 74 per cent and 162 per cent in Experiment 119. Thus corticosteroid addition increased the response only 6-fold and 2-fold, respectively, while in all other experiments hydrocortisone increased the basal response considerably more. These data suggest that the lymph nodes used in these two experiments contained a nearly adequate amount of the hormone (or its natural congener) for antibody production. Corticosteroid blood levels may have been elevated enough to saturate tissue sites if the donor rabbits were significantly stressed during their sacrifice by exsanguination. Stavitsky (10) has reported that "the mere handling of rabbits" occasionally produced lymphocytopenia, presumably due to adrenal cortical secretion. Therefore, in our more recent experiments rabbits have been killed quickly by a blow to the head early in the morning

before other work had commenced in the animal quarters. Sixteen experiments have been carried out with tissue from such rabbits; in only one did any response (18 per cent) occur in serum-free medium without hydrocortisone.

In most of these experiments the sodium hemisuccinate ester of hydrocortisone was used because of its high solubility and stability in aqueous solution. The succinate moiety does not contribute to the activity of the whole molecule

TABLE III

Responses to Hydrocortisone

Experiment No.	Concentration of hydrocortisone sodium hemisuccinate in μg/ml								
	0	0.0001	0.001	0.01	0.1	1.0	10	100	1000
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
103	0	—	—	—	23	**116**	79	—	—
106	2	—	—	20	20	**24**	18	17	—
109	7	—	5	—	—	**39**	—	0	0
113	0	—	6	7	**16**	**17**	0	0	0
119	74	—	41	74	**100**	**94**	**162**	108	—
122	4	6	28	121	**152**	—	**283**	106	0
128	0	—	1	36	**77**	—	**73**	4	—

The effect of different concentrations of hydrocortisone sodium hemisuccinate in Eagle's medium on the secondary antibody respoonse. For each experiment the response in Eagle's medium containing 25 per cent serum was the reference response and was arbitrarily expressed as 100 per cent. The percentage responses refer to anti-BSA for all experiments except No. 122, in which the antidiphtheria toxoid responses are recorded. The duration of the individual experiments varied from 15 to 21 days. (Other cultures in these experiments were maintained in hydrocortisone-containing Eagle's medium supplemented further with non-essential amino acids, insulin, and vitamin B_{12}. The responses with these additional supplements are not recorded in this table but were frequently greater than corresponding responses in Eagle's medium containing the same concentration of hydrocortisone.)

The maximum responses for each experiment are set in **boldface type** and were produced in medium containing 0.1 to 10 μg/ml of hydrocortisone (0.2 to 20 μM).

in supporting antibody production (Table IV). The responses obtained with hydrocortisone varied considerably in the three experiments shown in Table IV, undoubtedly because unsupplemented Eagle's medium was used in the first two experiments, while the third also included insulin, vitamin B_{12}, and the non-essential amino acids.

Other experiments have been carried out to determine whether antibody production can be supported *in vitro* by steroids structurally similar to hydrocortisone. Desoxycholate and tetrahydrocortisone do not produce any of the physiological effects characteristic of hydrocortisone. They are, therefore, often useful in establishing whether an observed biological response is due to the specific activity of a steroid being studied or to some property common to all compounds containing the steroid nucleus. Table V shows that in three experi-

TABLE IV

Hydrocortisone Compared with Serum and Succinic Acid

Experiment No.	Concentration of acid and hydrocortisone	Anti-BSA response in basal medium and +			
		No other supplement	Succinic acid	Hydrocortisone Na hemisuccinate	25 per cent normal rabbit serum
	μM	*per cent*	*per cent*	*per cent*	*per cent*
109	2	7	6	39	100
113	2	0	2	13	100
139	1	8	10	104	100

Secondary antibody responses in succinic acid compared to responses in equimolar concentrations of hydrocortisone sodium hemisuccinate. The basal medium consisted of only Eagle's medium in Experiments 109 and 113 but also included 0.1 mM each of the 7 non-essential amino acids and 1.0 unit/ml insulin in Experiment 139. For each experiment the response in Eagle's medium containing 25 per cent serum was the reference response and was arbitrarily expressed as 100 per cent. The duration of these experiments varied from 18 to 21 days.

TABLE V

Control Experiments with Inactive Steroids

Experiment No.	Steroid concentration	Antibody tested	Secondary response in basal medium and			
			No other supplements	Desoxycholate	Tetrahydrocortisone	Hydrocortisone Na hemisuccinate
	μM		*per cent*	*per cent*	*per cent*	*per cent*
109	2	BSA	19	5	—	100
		Diph.	34	9	—	100
139	1	BSA	8	19	17	100
		Diph.	1	2	4	100
146	1	BSA	0	—	1	100
		Dipt.	0	—	0	100

Secondary antibody response in desoxycholate and tetrahydrocortisone compared to responses in equimolar concentrations of hydrocortisone sodium hemisuccinate. The basal medium consisted of only Eagle's medium in Experiment 109 but also included 0.1 mM each of the 7 non-essential amino acids (*v.* Table I) and 1.0 unit/ml insulin in Experiment 139; 0.5 μg/ml vitamin B_{12} was added to these supplements in Eagle's medium for Experiment 146. For each experiment the response in the basal medium containing hydrocortisone was the reference response and was arbitrarily expressed as 100 per cent. The duration of these experiments varied from 18 to 21 days.

ments 1 to 2 μM of hydrocortisone greatly enhanced the antibody response, while equimolar concentrations of desoxycholate or tetrahydrocortisone had little or no effect.

Three different forms of hydrocortisone,—the free alcohol, the sodium

hemisuccinate, and phosphate esters,—as well as cortisone acetate and corticosterone support antibody production in the absence of serum. Table VI lists both the anti-BSA and antidiphtheria toxoid responses for three experiments in which equimolar concentrations of these five corticosteroids were tested. The 100 per cent response in each experiment was that produced in serum-free medium containing hydrocortisone sodium hemisuccinate. There is a fairly consistent correspondence between both responses to the same steroid in the

TABLE VI

Comparison between Hydrocortisone and Related Corticosteroids

Experiment No.	Steroid concentration	Antibody tested	Secondary antibody response in basal medium and					
			No supplement	Hydrocortisone Na hemisuccinate	Hydrocortisone phosphate	Cortisol (free alcohol of hydrocortisone)	Cortisone acetate	Corticosterone
	μM		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
109	2	BSA	19	100	43	—	55	—
		Diph.	34	100	73	—	70	—
139	1	BSA	8	100	—	130	158	274
		Diph.	1	100	—	83	165	184
146	1	BSA	0	100	—	75	141	143
		Diph.	0	100	—	53	88	141

Range of secondary antibody responses in equimolar concentrations of hydrocortisone derivatives, cortisone acetate, and corticosterone. The basal medium consisted of only Eagle's medium in Experiment 109 but also included 0.1 mM of each of the 7 non-essential amino acids and 1.0 unit/ml insulin in Experiment 139; 0.5 μg/ml vitamin B_{12} was added to these supplements in Eagle's medium for Experiment 146. For each experiment the response in basal medium containing hydrocortisone was the reference response and was arbitrarily expressed as 100 per cent. The duration of these experiments varied from 18 to 21 days.

same experiment. Furthermore, the range of responses in each experiment is similar for the three hydrocortisone derivatives and cortisone acetate, suggesting that the secondary response *in vitro* is promoted to essentially the same degree by similar concentrations of these four forms of 17-hydroxycorticosteroid.

In the rabbit the major corticosteroid normally secreted by the adrenal cortex is corticosterone rather than hydrocortisone (11, 12). In two experiments shown in Table VI equimolar concentrations of corticosterone appeared to produce higher responses than the other steroids tested. However, in another experiment (Fig. 6) a detailed comparison was made of the responses over a wide range of hydrocortisone sodium hemisuccinate and corticosterone concentrations; these data suggest that equimolar concentrations of the two support the secondary response *in vitro* to the same general extent. In this experiment the

average response of each similarly treated group of four cultures was calculated as the percentage of the response evoked in the presence of 1.0 μM of hydrocortisone.

Exposure to Hydrocortisone for Different Intervals during the Secondary Response in Vitro.—Fig. 7 shows the relative importance of supplementation with

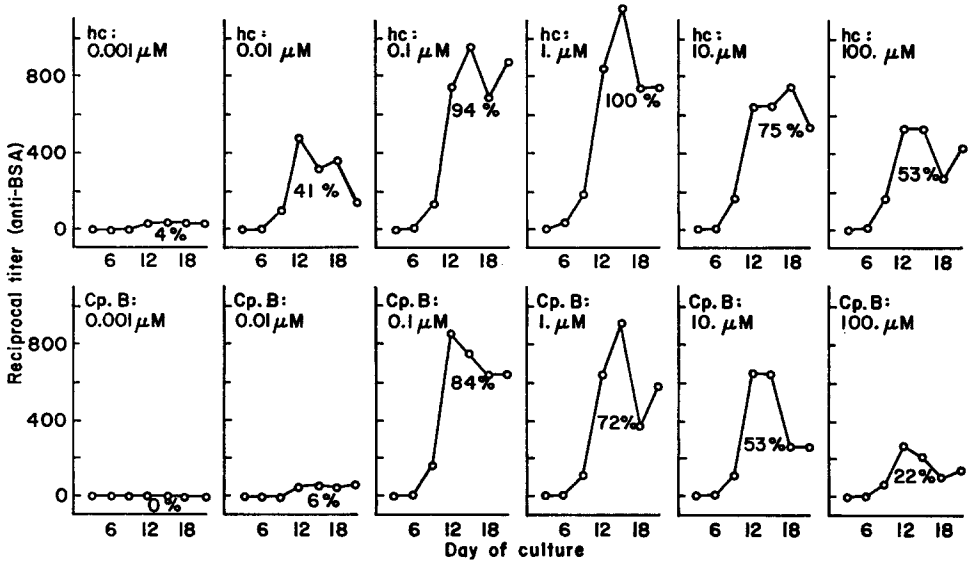


FIG. 6. The secondary antibody responses in a serum-free basal medium containing various concentrations of hydrocortisone sodium hemisuccinate (*hc*) or corticosterone (*Cp.B*). The basal medium consisted of Eagle's medium supplemented with 0.1 mM each of 6 "non-essential" amino acids (asparagine was omitted), 0.5 μg/ml vitamin B_{12}, and 0.5 units/ml insulin. The 100 per cent reference response was arbitrarily taken as the average response by those cultures maintained in basal medium containing 1.0 μM hydrocortisone.

0.01 μM hydrocortisone for various periods during the first 21 days of the secondary response. The presence of hydrocortisone in the medium for the first 6 or the first 9 days permitted responses of 55 per cent and 125 per cent, respectively. In other cultures the inclusion of hydrocortisone in the medium from day 6 until day 21 produced only a 13 per cent response and from day 9 until day 21 permitted only a 3 per cent response. These data reveal that hydrocortisone is required in the medium during the first 6 to 9 days after antigen stimulation *in vitro* for significant antibody responses. Although the cultures were exposed to the minimum effective concentration of hydrocortisone and although they were rinsed three times in steroid-free medium at the end of their various periods of hydrocortisone exposure (as indicated by the arrow in

each graph), this is no assurance that hydrocortisone does not persist and function within the cells producing antibody for some time afterward.

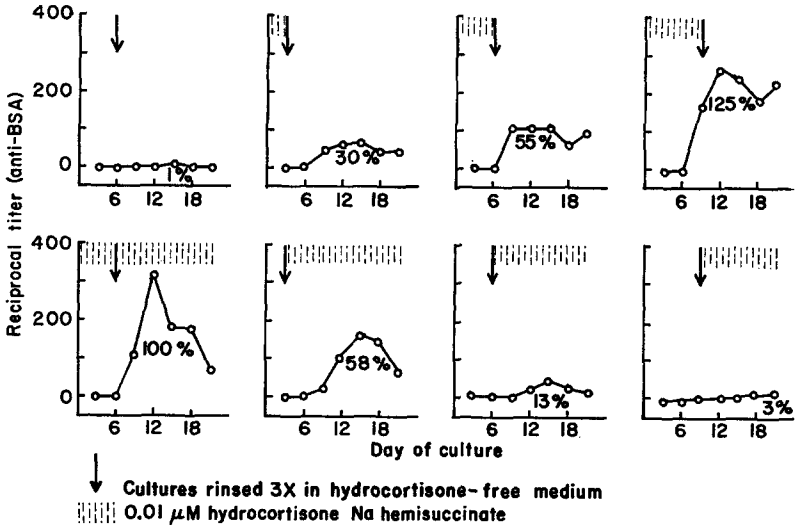


FIG. 7. The secondary antibody responses of cultures maintained in serum-free basal medium fortified with 0.01 μM hyrocortisone sodium hemisuccinate during the various intervals indicated by the stippled areas. Each culture was rinsed three times in hydrocortisone-free medium on the day indicated by the arrow. The 100 per cent reference response was the average response produced by those cultures exposed to hydrocortisone in their medium for 21 days. The basal medium consisted of Eagle's medium supplemented with 0.1 mM each of six nonessential amino acids (asparagine was excluded), 0.5 μg/ml vitamin B_{12}, and 0.5 units/ml insulin.

DISCUSSION

The development of a synthetic medium for a particular culture system has at least two aims. The immediately desired one is the more complete control of the system by elimination of biological fluids of unknown and often variable composition. But an ultimately more important gain is the elucidation of new metabolic relationships.

Early attempts to improve tissue culture media involved protein-free preparations of serum, such as ultrafiltrates and dialysates. In the late 1930's, Simms used ultrafiltrates to obtain tissue cultures free of fat granules and to prepare high-titered virus suspensions free of extraneous protein (13). Dialysates of serum were extensively studied by Fischer *et al.* (14), who ascribed their favorable effect to accessory growth substances. More recently, Eagle (15, 16) has given the same interpretation to his studies with dialyzed serum. In these experiments Eagle used a culture vessel consisting of two compartments separated by a cellophane membrane. When a cell sus-

pension in Eagle's medium in one compartment was separated by the membrane from medium containing 5 per cent dialyzed serum, growth did not occur. However, addition of a pancreatic extract to the serum side of the vessel stimulated growth of the cell suspension on the opposite side. Eagle suggested that the enzyme released "essential growth factors of small molecular weight" from the previously dialyzed serum.

In related studies with isolated L cells, Gwatkin (17) found that a chemically defined medium (CMRL-1066) supported cell growth as well when it was supplemented by dialysis against horse serum for 24 hours as it did when it contained 20 per cent whole serum. Analogous results with other cell strains were reported by Metzgar and Moskowitz (18), who dialysed medium 1066 for 48 hours against horse serum which had previously been incubated at 37°C for 7 days or longer.

Among the dialyzable components of serum most carefully studied for their binding properties to proteins have been the corticosteroids. At normal serum levels in man cortisol and corticosterone are bound chiefly to an alpha-1-globulin, designated "corticosteroid-binding globulin" by Daughaday (19) and "transcortin" by Slaunwhite and Sandberg (20). Substantial concentrations of transcortin have been found in the serum of rabbits and other laboratory animals (20, 21). Concentrations of the two hormones above the normal physiological level in the serum are loosely bound to albumin (22). These and other quantitative observations have been obtained by precise equilibrium dialysis techniques, which resemble the simple dialysis procedures used in the tissue culture experiments previously described.

Correlations between these two sets of investigations suggest that corticosteroids or related steroids were responsible for the effects noted in the tissue culture experiments with dialyzed serum. First, at 37°C the strength of the cortisol-transcortin bond is about half that observed at 4°C (23), but the binding of cortisol and other steroids to human serum albumin is not weakened by a corresponding temperature rise (20). In the tissue culture studies of Metzgar and Moskowitz (18) dialysis of medium 1066 against horse serum at 37°C enriched this medium far more than a similar dialysis at 4°C. Secondly, Slaunwhite and Sandberg (20) found that overnight digestion of transcortin with trypsin completely destroyed the cortisol-binding property. In Eagle's experiment the pancreatic extract may have produced the same effect and released various steroids from the previously dialyzed serum. Also in Metzgar and Moskowitz's study the greater enrichment obtained at 37°C may have been partly due to more complete proteolysis at this temperature than at 4°C.

Thus certain physical properties of the corticosteroid-transcortin complex are compatible with the possibility that cortisol or a similar steroid produced the favorable effects observed in the tissue culture studies reviewed above. Also consistent with this interpretation is our observation that a serum dialysate extracted with chloroform did not support antibody production *in vitro*. However, the most convincing evidence is the fact that corticosteroids alone duplicate the effects of serum dialysates in supporting antibody production in our cultures.

Effects of Adrenal Cortical Hormones on Tissue Cultures.—Tissue culture experiments involving corticosteroids are discussed in this section according to

the effects noted (*a*) on the outgrowth from tissue explants, (*b*) on more exact assays of cell multiplication, and (*c*) on cell survival *in vitro*. With few exceptions these hormones have been reported to decrease the growth and shorten the survival of cultures.

Since both cell migration and cell multiplication contribute to the initial *outgrowth from primary cultures*, measurement of outgrowth reflects several cellular effects of these hormones. Ketchel *et al.* (24) studied the inhibition of human leukocyte migration in capillary tubes and found that migration decreased in proportion to the concentration of hydrocortisone sodium succinate above 0.1 μg/ml. In our cultures in which Eagle's medium with serum was replaced by serum-free medium containing hydrocortisone there was almost complete absence of outgrowth from the lymph node fragments. Despite this, antibody production was well supported. Hence, outgrowth measurements do not always parallel other parameters of cell function. For this reason reports based on such measurements will not be discussed further.

In more precise studies on *cell multiplication* several groups of investigators (25–27) have reported reduced mitotic counts in various human cells cultured with corticosteroids. The effect of these steroids for 3 or more days has also been measured by means of whole cell counts or enumeration of cell nuclei. Mouse lymphoma cells are very sensitive to hydrocortisone, since 0.1 μM reduced cell counts 50 per cent (28). The growth of Earle's L cells, a strain of mouse fibroblast, was inhibited 50 per cent by 10 μg/ml (29) or 35 μg/ml (30). Bass and Snell (26) observed a 28 per cent reduction in HeLa cell nuclear counts with 50 μg/ml of hydrocortisone sodium succinate. Using the same compound, Fand and Orlan (31) observed no effect from 50 μg/ml on the number of viable HeLa and Kb cells; however, 1 μg/ml caused a 59 per cent reduction in counts of cultures of human adult sternal marrow (D98S).

These and other studies not cited here indicate a wide range of sensitivity of different cultures to the inhibitory effect of corticosteroids. It has been suggested that cell strains can be classified as corticosteroid-sensitive or insensitive on the basis of their embryonic origin (31). In most of these reports inhibition was produced by steroid concentrations far higher than those occurring normally in the blood. Little consideration appears to have been given the possibility that physiological levels of these hormones may enhance cell growth, since in most reports cell counts from cultures containing such levels were not listed.

The effect of corticosteroids on *cell survival* was initially studied in lymphocytes during incubation periods of 2 days or less. The survival time of rabbit thymocytes (32) and rat lymphocytes (33) was strikingly reduced by these drugs; as little as 0.06 μg/ml of hydrocortisone halved the survival of rabbit thymocytes. In contrast, Rosenau and Moon (34) observed no decrease in survival of mouse spleen lymphocytes cultured in 125 μg/ml of hydrocortisone.

The influence of steroids on survival of other cell types has only recently been investigated and has led to conclusions different from the earlier lymphocyte studies. A notable report by Gillette *et al.* (35) concerns the survival of full thickness mouse skin

in medium containing cortisone acetate. Survival during 4 weeks of culture was assayed by grafting both control and steroid-treated skin fragments back into their original donors at weekly intervals. None of the grafts from 3-week-old control cultures survived; however, 83 per cent of the grafts from 3-week-old steroid-treated cultures were successful. During culture of the untreated fragments, "primitive cells" grew out onto the surface of the culture flask and necrotic cells increased within the fragments. Such outgrowths were not found in the treated cultures, and necrotic cells and other degenerative changes appeared only weeks later. The authors concluded that cortisone retarded "the modulation of cells" into more primitive, non-functional forms which readily migrate out of the fragments and which thereby initiate "the disorganizing process" seen much earlier in control cultures.

A related report by Fand (36) concerns the effect of hydrocortisone on an established cell strain derived from human sternal marrow (D-98). The plating efficiency of a trypsinized suspension was increased nearly 3-fold by 0.5 to 5.0 μg/ml of hydrocortisone in the medium. Fand suggested that the hormone prevented damage by trypsin to the cell membrane.

A very different protective effect has been proposed for hydrocortisone in other injured cells. Cultures of various rabbit and rat cells release acid hydrolases from lysosomes when they are exposed to ultraviolet irradiation (37) or to both excess vitamin A and ultraviolet radiation (38). Rat fetal skin cultures treated with 7.5 μg/ml of hydrocortisone for 2 days before irradiation showed fewer degenerative changes than the untreated controls. This steroid-protective effect was attributed to the stabilization of lysosomes (37, 38).

The last three groups of investigations cited above have no obvious relationship to our studies on antibody formation except that all describe an apparently "favorable" influence of corticosteroid hormones on certain aspects of cellular metabolism, whether they relate to survival, multiplication, or specific protein synthesis. In several experiments we have compared the duration of antibody production by cultures maintained in medium containing serum or in serum-free medium containing low levels of hydrocortisone; in these experiments the media were replaced every 3 days. The latter cultures invariably produced higher titers for longer periods. This suggests that although serum can initiate and support the secondary response *in vitro*, it may exert an inhibitory effect in time.

These sets of cultures also differed morphologically. As described previously, fragments maintained in the hydrocortisone-fortified medium showed little or no outgrowth. This inhibition of outgrowth was probably due primarily to the absence of serum in the medium; however, hydrocortisone may also have exerted a positive influence in this direction. Fell (39) has stated that "the great enemy of the organ culturist is . . . emigration." This undesired process not only accompanies but undoubtedly promotes the disorganization and dedifferentiation and the ensuing loss of function typical of many tissues during their cultivation.

Pituitary-Adrenal Influences on the Immune Response.—From an immunological viewpoint the effect of adrenocortical hormones on antibody production *in vivo* has been exhaustively investigated. However, the results reported have been remarkably divergent, and the attempts to resolve them on the basis of species differences (40) or antigen particle size (41, 42) are not thoroughly convincing. Some indication of the confusion in this area may be obtained from the following paragraph which summarizes the more relevant investigations.

This problem was initially studied in adrenalectomized animals. When such animals were subsequently injected with an antigen, enhanced antibody responses were usually observed (43–47). However, in other studies the response was either unchanged (48–50) or depressed (51, 52). The 1928 study of Perla and Marmorston-Gottesman (51) is regularly cited as an example of the inhibitory effect of adrenalectomy; however, these authors *also* reported that injection of "ten times the normal optimum antigen amount" produced higher antibody responses than were obtained in their unoperated animals. The next approach was the injection of adrenal cortical extracts prior to and during antigen stimulation. Paradoxically, this was generally associated with enhanced antibody responses (53–55, 45), although Eisen *et al.* (49) found no difference in serum precipitin levels of control and extract-treated adrenalectomized rats after immunization with pneumococcal polysaccharide. In recent years ACTH, cortisone, and hydrocortisone have been widely tested and were reported by most investigators to depress the immune response (*cf.* reviews by Mirick, reference 40, Fagraeus, reference 56, and Ward and Johnson, reference 57). In 1955 Newsom and Darrach (58) showed that corticosterone also suppressed the normal rise of circulating hemolysin to sheep erythrocytes in mice. (In the same year Kass *et al.*, reference 59 reported that corticosterone did not reduce the antibody response in rabbits; however, recently Kass, reference 60 has explained these negative results as due to inadequate absorption of this steroid from injection sites.) Three groups of workers have shown that cortisone suppression of the immune response can be overcome by increasing the antigen dosage at least 50-fold (61, 62, 57). In the future, consideration of the corticosteroid-antigen ratio may help resolve some of these discrepancies.

More closely related to our own organ culture work are several *in vitro* studies on the influence of adrenal cortical hormones. Roberts and White (63) found that the antibody content of mesenteric nodes, spleen, and thymus extracts reached a peak level 5 days after intravenous antigen injection in normal rats. Adrenalectomy prior to immunization delayed and depressed the antibody rise in lymph nodes and thymus. Spleen minces, incubated in Warburg flasks for 3 hours, released antibodies into the medium. This release was greatest for spleens obtained from rats treated with ACTH or adrenal cortical extract and least for spleens from adrenalectomized rats. These data plus the titers of pre- and postincubation spleen extracts, suggested that new antibody was formed during the 3 hour incubation by spleens from control (nontreated) and hormone-injected rats but not by spleens from adrenalectomized rats.

In a similar report, van der Slikke and Keuning (64) measured agglutinin titers of extracts of red and white pulp of rabbit spleen before and after incubation for 20 hours in rabbit serum–Tyrode medium. The difference in the titers indicated antibody

production *in vitro*. Cultures prepared from rabbits injected 24 hours previously with ACTH showed titers more than double those of cultures from non-injected rabbits.

Spleen fragments from immunized but otherwise normal rabbits have been exposed to cortisone during culture by several workers. Fagraeus (56) noted that 70 μg/ml had no effect on the antibody-producing capacity of her cultures, while Mountain (65) reported that 320 μg/ml or more depressed the antibody response *in vitro* during a 48 hour period.

The *in vivo* reports summarized in a preceding paragraph are not only inconsistent with one another but also incompatible with the *in vitro* studies listed immediately above. For example, prior adrenalectomy appears to impair antibody synthesis *in vitro* yet generally enhances the immune response *in vivo*. Furthermore, prior treatment with ACTH promotes antibody synthesis in cultures yet generally suppresses the immune response in the intact animal. Unfortunately, none of the four *in vitro* studies is comparable enough to our own organ culture experiments to justify any attempts to correlate them. The former were performed on tissues in which the immune response was well established, if not already waning. In our experiments the secondary response was initiated *in vitro*, and the entire sequence of metabolic steps from the assimilation of antigen to the release of antibody could be experimentally manipulated to some degree during a period of several weeks. Nevertheless, all *in vitro* work, including ours and that of others, is consistent with the conclusion that low levels of corticosteroids are necessary for the promotion of antibody production.

In the majority of the reports cited throughout this discussion, investigators have employed concentrations of corticosteroids many times greater than that normally found in the circulation. This consideration has evoked the suggestion that such reports really concern the "pharmacological" effects of these steroids. Although the levels of these hormones at their target sites in tissues are unknown (66), it is generally accepted that "physiological" concentrations approximate the usual blood levels (67). In normal rabbits van der Vies (12) found in peripheral plasma hydrocortisone levels of 0.06 to 0.17 μM and corticosterone levels of 0.14 to 0.29 μM. From rabbits injected daily with ACTH for 3 or 4 weeks Kass *et al.* (11) obtained adrenal vein blood and found hydrocortisone levels of 8.3 μM and corticosterone levels of 4.3 μM. Our observations that the secondary antibody response *in vitro* is supported by 0.01 to 1.0 μM of hydrocortisone sodium hemisuccinate or 0.1 to 1.0 μM of corticosterone suggest that this function represents a physiological, and not a pharmacological, effect of these hormones.

SUMMARY

It was previously reported from this laboratory that the secondary antibody response can regularly be elicited *in vitro* from fragments of rabbit lymph node node cultured in Eagle's medium supplemented with normal rabbit serum.

Evidence is now presented that physiological levels of hydrocortisone (0.01 to 1.0 μM) can substitute for serum in the culture medium. However, with the omission of serum, serine (0.1 mM) must be included among Eagle's "essential" amino acids for consistent optimal antibody production. In some experiments the addition of insulin (0.5 unit/ml) and vitamin B_{12} (0.5 μg/ml) has further enhanced the secondary response in this serum-free medium.

I am most grateful for the generous support and continued encouragement of Dr. Albert H. Coons and during the initiation of this work for valuable discussions with Dr. John G. Harter. The cheerful and proficient technical assistance of Mrs. Linda Santoro throughout these studies and that of Miss Shirley Fishman, Mr. Peter M. Fitz, and Mrs. Judy Kolk at different times is a pleasure to acknowledge. I am also indebted to Dr. Paul W. O'Connell of Parke, Davis and Company, Detroit for his gifts of hydrocortisone sodium hemisuccinate (solu-cortef) and to Dr. William J. Reddy and Dr. W. H. Pearlman for samples of several other corticosteroids tested.

BIBLIOGRAPHY

1. Michaelides, M. C., and Coons, A. H., Studies on antibody production. V. The secondary response *in vitro*, *J. Exp. Med.*, 1963, **117**, 1035.
2. Ambrose, C. T., and Coons, A. H., The replacement of serum by hydrocortisone in the production of antibody *in vitro*, *Fed. Proc.*, 1963, **22**, 266.
3. Ambrose, C. T., and Coons, A. H., Studies on antibody production. VIII. The inhibitory effect of chloramphenicol on the synthesis of antibody in tissue culture, *J. Exp. Med.*, 1963, **117**, 1075.
4. Stavitsky, A. B., Micromethods for the study of proteins and antibodies. I. Procedure and general application of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells, *J. Immunol.*, 1954, **72**, 360.
5. Parker, R. C., Methods of Tissue Culture, New York, Paul B. Hoeber, Inc., 1961, 62.
6. Puck, T. T., Cieciura, S. J., and Robinson, A., Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects, *J. Exp. Med.*, 1958, **108**, 945.
7. Eagle, H., Amino acid metabolism in mammalian cell cultures, *Science*, 1959, **130**, 432.
8. Kabat, E. A., Kabat and Mayer's Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961, 867.
9. Levinthow, L., Eagle, H., and Piez, K. A., The role of glutamine in protein biosynthesis in tissue culture, *J. Biol. Chem.*, 1957, **227**, 929.
10. Stavitsky, A. B., The influence of adrenal cortical extract on immunological phenomena *in vivo*. I. Antigen-antibody reactions as indicated by hypocomplementemia, granulocytopenia and reduction of circulating antibody, *J. Immunol.*, 1952, **69**, 63.
11. Kass, E. H., Hechter, O., Macchi, I. A., and Mou, T. W., Changes in patterns of secretion of corticosteroids in rabbits after prolonged treatment with ACTH, *Proc. Soc. Exp. Biol. and Med.*, 1954, **85**, 583.

12. van der Vies, J., Individual determination of cortisol and corticosterone in a single small sample of peripheral blood, *Acta Endocrinol.*, 1961, **38,** 399.
13. Simms, H. S., and Sanders, M., Use of serum ultrafiltrate in tissue cultures for studying deposition of fat and for propagation of viruses, *Arch. Path.*, 1942, **33,** 619.
14. Fischer, A., Astrup, T., Ehrensvard, G., and Oehlenschlager, V., Growth of animal tissue cells in artificial media, *Proc. Soc. Exp. Biol. and Med.*, 1948, **67,** 40.
15. Lockart, R. Z., Jr., and Eagle, H., Requirements for growth of single human cells, *Science*, 1959, **129,** 252.
16. Eagle, H., The sustained growth of human and animal cells in a protein-free environment, *Proc. Nat. Acad. Sc.*, 1960, **46,** 427.
17. Gwatkin, R. B., Are macromolecules required for growth of single isolated mammalian cells? *Nature*, 1960, **186,** 984.
18. Metzgar, D. P., and Moskowitz, M., Separation of growth promoting activity from horse serum by dialysis, *Proc. Soc. Exp. Biol. and Med.*, 1960, **104,** 363.
19. Daughaday, W. H., Binding of corticosteroids by plasma proteins. IV. The electrophoretic demonstration of corticosteroid binding globulin, *J. Clin. Inv.*, 1958, **37,** 519.
20. Slaunwhite, W. R., Jr., and Sandberg, A. A., Transcortin: a corticosteroid-binding protein of plasma, *J. Clin. Inv.*, 1959, **38,** 384.
21. Seal, U. S., and Doe, R. P., Corticosteroid-binding globulin: species distribution and small-scale purification, *Endocrinology*, 1963, **73,** 371.
22. Daughaday, W. H., Binding of corticosteroids by plasma proteins. III. The binding of corticosteroid and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis, *J. Clin. Inv.*, 1958, **37,** 511.
23. Daughaday, W. H., and Mariz, I. K., Corticosteroid-binding globulin: its properties and quantitation, *Metabolism*, 1961, **10,** 936.
24. Ketchel, M. M., Favour, C. B., and Sturgis, S. H., The *in vitro* action of hydrocortisone on leukocyte migration, *J. Exp. Med.*, 1958, **107,** 211.
25. Wellington, J. S., and Moon, H. D., The effect of hydrocortisone on cultures of human liver cells, *Fed. Proc.*, 1960, **19,** 158.
26. Bass, A. D., and Snell, C. C., Action of hydrocortisone sodium succinate on HeLa cells, *J. Pharm. and Exp. Therap.*, 1961, **134,** 83.
27. Nowell, P. C., Inhibition of human leukocyte mitosis by prednisolone *in vitro*, *Cancer Research*, 1961, **21,** 1518.
28. Gabourel, J. D., and Aronow, L., Growth inhibitory effects of hydrocortisone on mouse lymphoma ML-388 *in vitro*, *J. Pharm. and Exp. Therap.*, 1962, **136,** 213.
29. Holden, M., and Adams, L. B., Inhibitory effects of cortisone acetate and hydrocortisone on growth of fibroblasts, *Proc. Soc. Exp. Biol. and Med.*, 1957, **95,** 364.
30. Perlman, D., Giuffre, N. A., Brindle, S. A., and Pan, S. C., Cytotoxicity of steroids to mammalian cells in tissue culture, *Proc. Soc. Exp. Biol. and Med.*, 1962, **111,** 623.
31. Fand, I., and Orlan, M., Effects of 17-hydroxycorticosterone in distinguishing mammalian clone cultured cells of similar morphology, *J. Morphol.*, 1962, **111,** 89.

32. Schrek, R., Cytotoxic action of hormones of the adrenal cortex according to the method of unstained cell counts, *Endocrinology*, 1949, **45,** 317.
33. Trowell, O. A., The action of cortisone on lymphocytes *in vitro*, *J. Physiol.* 1953, **119,** 274.
34. Rosenau, W., and Moon, H. D., The inhibitory effect of hydrocortisone on lysis of homologous cells by lymphocytes *in vitro*, *J. Immunol.*, 1962, **89,** 422.
35. Gillette, R. W., Findley, A., and Conway, H., Effect of cortisone on skin maintained in organ culture, *J. Nat. Cancer Inst.*, 1961, **27,** 1285.
36. Fand, I., Increased plating efficiency following application of hydrocortisone in mammalian cell clone cultures, *Fed. Proc.* 1962, **21,** 159.
37. Weissmann, G., and Fell, H. B., The effect of hydrocortisone on the response of fetal rat skin in culture to ultraviolet irradiation, *J. Exp. Med.*, 1962, **116,** 365.
38. Weissmann, G., and Thomas, L., Studies on lysosomes. II. The effect of cortisone on the release of acid hydrolases from a large granule fraction of rabbit liver induced by an excess of vitamin A, *J. Clin. Inv.*, 1963, **42,** 661.
39. Fell, H. B., Discussion, *in* Decennial Review Conference on Tissue Culture, *J. Nat. Cancer Inst.*, 1957, **19,** 601.
40. Mirick, G. S., The effects of ACTH and cortisone on antibodies in human beings, *Bull. Johns Hopkins Hosp.*, 1951, **88,** 332.
41. Hanan, R., and Oyama, J., Particle size of antigen in relation to antibody-suppressive effect of cortisone, *Proc. Soc. Exp. Biol. and Med.*, 1954, **85,** 373.
42. Berglund, K., Studies on factors which condition the effect of cortisone on antibody production. IV. The significance of the particle size of the antigen, *Acta Path. et Microbiol. Scand.*, 1956, **39,** 41.
43. Také, N. M., and Marine, D., The effect of suprarenalectomy in rabbits on hemolysin formation, *J. Infect. Dis.* 1923, **33,** 217.
44. Jaffe, H. L., and Marine, D., Effect of suprarenalectomy in rats on agglutinin formation, *J. Infect. Dis.*, 1924, **35,** 334.
45. Murphy, J. B., and Sturm, E., The lymphoid tissue and antibody formation, *Proc. Soc. Exp. Biol. and Med.*, 1947, **66,** 303.
46. Criep, L. H., Mayer, L. D., and Menchaca, O. E. L., The effect of adrenalectomy on experimental hypersensitiveness, *J. Allergy*, 1951, **22,** 314.
47. Char, D. F. B., and Kelley, V. C., Serum antibody and protein studies following adrenalectomy in rabbits, *Proc. Soc. Exp. Biol. and Med.*, 1962, **109,** 599.
48. Khorazo, D., Agglutinin production in suprarenalectomized rats, *J. Immunol.*, 1931, **21,** 151.
49. Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R.-R., and Stoerk, H. C., Failure of adrenal cortical activity to influence circulating antibodies and gamma globulin, *Proc. Soc. Exp. Biol. and Med.*, 1947, **65,** 301.
50. Thatcher, J. S., Houghton, B. C., and Ziegler, C. H., Effect of adrenalectomy and adrenal cortical hormone upon the formation of antibodies, *Endocrinology*, 1948, **43,** 440.
51. Perla, D., and Marmorston-Gottesman, J., Immunological studies in relation to the suprarenal gland. II. Hemolysin formation in suprarenalectomized rats, *J. Exp. Med.*, 1928, **47,** 723.

52. Chase, J. H., The effect of adrenalectomy upon the maintenance of titer to sheep erythrocytes in hyperimmunized mice, *Bull. New York Med. Coll. Flower and Fifth Ave. Hosp.*, 1947, **10,** 13.
53. Fox, C. A., and Whitehead, R. W., Relation of the adrenal glands to immunological processes. Effect of cortico-adrenal extract on hemolysin production in normal adult laboratory animals, *J. Immunol.*, 1936, **30,** 51.
54. Daugherty, T. F., White, A., and Chase, J. H., Relationship of the effects of adrenal cortical secretion on lymphoid tissue and on antibody titer, *Proc. Soc. Exp. Biol. and Med.*, 1944, **56,** 28.
55. Chase, J. H., White, A., and Dougherty, T. F., The enhancement of circulating antibody concentration by adrenal cortical hormones, *J. Immunol.*, 1946, **52,** 101.
56. Fagraeus, A., Role of ACTH and cortisone in resistance and immunity, *Acta Path. et Microbiol. Scand.*, 1952, **93,** 20.
57. Ward, P. A., and Johnson, A. G., Studies on the adjuvant action of bacterial endotoxins on antibody formation. II. Antibody formation in cortisone-treated rabbits, *J. Immunol.*, 1959, **82,** 428.
58. Newsom, S. E., and Darrach, M., The effect of corticotropin and corticosterone on the production of hemolytic antibodies in the mouse, *Canad. J. Biochem. and Physiol.*, 1955, **33,** 374.
59. Kass, E. H., Hechter, O., Mou, T. W., and Lurie, M. B., Effects of adrenal steroids on resistance to infection, *Arch. Int. Med.*, 1955, **96,** 397.
60. Kass, E. H., General discussion, *in* Mechanisms of Hypersensitivity, (J. H. Shaffer, G. A. LoGrippo, and M. W. Chase, editors), Boston, Little, Brown and Co., 1959, 633.
61. Newsom, S. E., and Darrach, M., The effect of cortisone acetate on the production of circulating hemolytic antibodies in the mouse, *Canad. J. Biochem. and Physiol.*, 1954, **32,** 372.
62. Berglund, K., Studies on factors which condition the effect of cortisone on antibody production. II. The significance of the dose of antigen in primary hemolysin response, *Acta Path. et Microbiol. Scand.*, 1956, **38,** 329.
63. Roberts, S., and White, A. The influence of the adrenal cortex on antibody production *in vitro*, *Endocrinology*, 1951, **48,** 741.
64. van der Slikke, L. B., and Keuning, F. J., Influence of adrenocortical stimulation on antibody production, *J. Lab. and Clin. Med.*, 1953, **42,** 389.
65. Mountain, I. M., Antibody production by spleen *in vitro*. I. Influence of cortisone and other chemicals, *J. Immunol.*, 1955, **74,** 270.
66. Villee, C. A., The hormonal regulation of metabolic processes, and Discussion, *in* Mechanism of Action of Steroid Hormones, (C. A. Villee and L. L. Engel, editors), New York, Pergamon Press, 1961, 11 and 16.
67. Bush, I. E., Actions of adrenal steroids at cellular level, *Brit. Med. Bull.*, 1962, **18,** 141.