showed no inhibition in subsequent staining with the labelled anti-human globulin. This proves that the staining observed originally was due to the reaction of anti-human globulin with human globulins in the section.

Whether the slides were fixed in formalin‖ or acetone or not fixed at all, there was a definite precipitate formation when the unlabelled antihuman gamma globulin was added. The precipitate formed in solution and was removed on subsequent washing. The fact that staining was obtained in the presence of the precipitate forming in the suspension would indicate here also that there was excess antibody present and perhaps that the components which are being stained in the tissue may be of a different nature than the material precipitating.

Summary. 1) A new fluorescent label, tetramethylrhodamine isocyanate, is described and its use demonstrated in the immunohistochemical fluorescence technic. The label fluoresces orange and is of particular value for staining tissues which show a green autofluorescence similar to fluorescein fluorescence even when unstained. 2) Serum from a patient with chronic thyroiditis combined with normal thyroid tissue in the colloid region and perhaps in the glandular epithelium, while normal human serum showed no such staining. Thyroid from a patient with chronic thyroiditis, infiltrated with lymphocytic cells, was stained by rabbit antibody to human globulin in the region occupied by the infiltrating cells. Normal lymph node showed no such staining. This indicates that the infiltrating cells contained human globulin and may have been important in the destruction of the thyroid during the course of the disease.

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‖ The fixation process did not render the antigens completely insoluble.

1. Coons, A. H., *Internat. Rev. Cytol.,* 1956, v5, 1.
2. Clayton, R. M., *Nature,* 1954, v174, 1059.
3. Silverstein, A. M., *J. Histochem.,* 1957, v5, 94.
4. Roitt, I. M., Doniach, D., Campbell, P. N., Hudson, R. V., *Lancet,* 1956, v2, 820.
5. Witebsky, E., Rose, N. R., Terplan, K., Paine, J. R., *J. Am. Med. Assn.,* 1957, v164, 1439.
6. Witebsky, E., Rose, N. R., *J. Immunol.,* 1956, v76, 408.
7. Rose, N. R., Witebsky, E., *ibid.,* 1956, v76, 417.
8. Beutner, E. H., Witebsky, E., Rose, N. R., Gerbasi, J. R., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, in press.
9. White, R. G., *Proc. Royal Soc. of Med.,* 1957, v50, 953.
10. Pressman, D., James, A. W., Yagi, Y., Hiramoto, R., Woernley, D., Maxwell, W. T., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 773.
11. Freund, V., *J. Immunol.,* 1955, v75, 454.
12. Coons, A. H., Kaplan, M. H., *J. Exp. Med.,* 1950, v91, 1.
13. Hiramoto, R., Pressman, D., *Cancer Res.,* 1957, v17, 1135.

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Role of 11β-Hydroxyprogesterone as Intermediary in Biosynthesis of Cortisol and Corticosterone.* (23823)

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There is considerable evidence that production of 17α-hydroxycorticosterone (cortisol) and corticosterone from cholesterol in the adrenal cortex proceeds *via* the reaction scheme illustrated in Fig. 1(1,2). The indicated sequence of hydroxylations of progesterone, shown by solid arrows, has been re-

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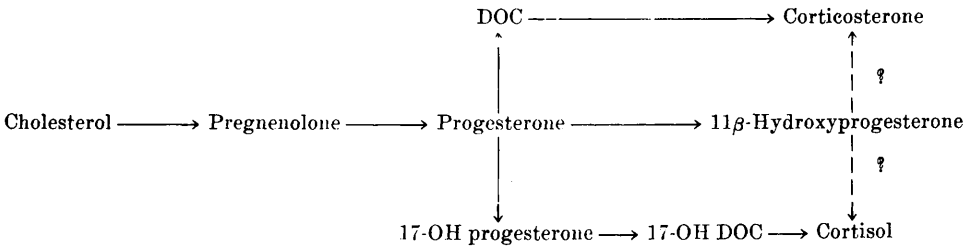

FIG. 1. Corticosteroidogenic Sequence.

garded as obligatory, and was postulated as the major route of biosynthesis of these corticoids. As indicated in Fig. 1, there is uncertainty as to the possible role of 11β-hydroxyprogesterone (HP) as an intermediary in this sequence. Although progesterone is converted in adrenal tissue to 11β-HP, the latter was originally considered not to be a *major* intermediary on the basis of adrenal perfusion experiments wherein 11β-HP failed to give rise to significant amounts of cortisol or corticosterone(3). However, these perfusion data do not permit a definitive conclusion, since adequate transfer of 11β-HP from the perfusate into the cells may not have occurred, whereas 11β-HP formed intracellularly might serve as an intermediary. The more recent findings that adrenal mitochondrial systems(4,5) convert progesterone to 11β-HP in good yield have served to reopen consideration of 11β-HP as a possible intermediary in corticosteroidogenesis.

To eliminate the influences of permeability barriers inherent in the perfusion technic, we have employed bovine adrenocortical homogenates, which synthesize corticoids from endogenous precursors, to reinvestigate the role of 11β-HP as an intermediary in corticoid biosynthesis. Tracer amounts of 11β-HP-4-C^{14} were incubated in homogenates prepared in various media, and the degree of incorporation into the cortisol and corticosterone formed was measured relative to that of comparable experiments with tracer amounts of progesterone-C^{14} (labelled either at C-4 or C-21) and DOC-21-C^{14}. The results of these studies demonstrate that while 11β-HP may be converted to both cortisol and corticosterone, comparison of the rates of conversion relative to progesterone and DOC indicate that in bovine adrenal tissue, 11β-HP is not a *major* intermediary in the biosynthesis of cortisol or corticosterone from progesterone.

Methods. A. *Preparation of homogenates.* Bovine adrenal cortex tissue was homogenized in each of the following media, using procedures previously described in detail(6): (a) 0.025M sucrose, (b) 0.154M KCl and (c) 0.154M NaCl. All media were buffered with M/150 Sorensen's phosphate (pH 7.2) and supplemented with niacinamide (.005M), sodium fumarate (.005M), $MgSO_4$ (.005M), adenosine triphosphate (ATP) (.001M) and diphosphopyridine nucleotide (DPN) (.0005M). B. *Steroid substrates.* The C^{14}-progesterone (S.A. 1.60 x 10^6 counts/min./mg) and DOC-21-C^{14} (S.A. 1.55 x 10^6 counts/min./mg) employed in these studies were highly purified preparations and regarded as radiochemically homogenous on the basis of our previous studies(6). The 11β-HP-4-C^{14} employed was a highly purified crystalline sample, kindly supplied by Dr. Harold Levy of these institutions, and was derived from bovine adrenal perfusion of progesterone-4-C^{14}. The sample had been fully characterized by Dr. Levy on the basis of standard organic technics as well as by I.R. Upon subjecting this sample to paper chromatography using a ligroin-toluene (1:1)/propylene glycol system, a modification of Zaffaroni(7), a single steroid zone was detected. Upon subsequent chromatography on silica gel columns, eluting with large volumes of a benzene-ethyl acetate (10:1) mixture, a single sharp symmetrical peak of radioactivity was observed which corresponded to the 11β-HP and contained more than 99% of the radioactivity. The 11β-HP employed had an original specific activity (S.A.) of 6.58 x 10^6 c./m./mg and a portion of this sample was diluted to S.A. of 1.55 x 10^6

c. m. mg. C. *Incubation conditions.* Homogenate aliquots corresponding to 35 to 100 g tissue were added to reaction vessels containing the radioactive steroid suspended in 0.3 ml propylene glycol, and were incubated for 2 hr with shaking (gas phase, 100% O_2). The radiosteroids were present in the various experiments in ratios ranging from 1.1 to 4.6 μM per 100 g equivalents of tissue (equivalent to 400 ml homogenate). After incubation the reactions were halted by the addition of 2 volumes of acetone. In each experiment aliquots of the same homogenate extracted without incubation served to measure the cortisol and corticosterone originally present in the homogenate, and thus permitted measurement of the net synthesis of these corticoids during the 2 hr period of incubation. D. *Extraction and isolation of corticoids.* The procedure employed has been previously described in detail(6). Briefly, this consisted of exhaustive extraction of homogenates with acetone and ethyl acetate, removal of the bulk of phospholipides by acetone-$MgCl_2$ precipitation in the cold, followed by fractionation on silica gel columns. The radioactive fractions eluted from the column were then divided into 2 equal portions, one of which was reserved for the procedures described in "E". The other portion was subjected to paper chromatography and the corticosterone and cortisol zones were cut out, eluted and rechromatographed. These steroids were then acetylated and subjected to 2 successive paper chromatograms on systems suitable for the monoacetates. At this stage of fractionation the corticosterone acetate and cortisol acetate were considered to be sufficiently pure to provide meaningful results concerning incorporation of C^{14}-substrates. The samples were counted (infinite thinness, using a flow-gas counter) and then quantitatively estimated using blue tetrazolium in a modified Mader-Buck procedure. E. *Radiochemical homogeneity of the corticosteroids.* This was assessed by determining the S.A. of the corticosteroids at various stages of purification. For this purpose, the portions of extracts remaining (50%) were appropriately pooled as described in the discussion of results. The chromatography of the steroid alcohols and monoacetates, as described above, was repeated on these pooled samples and the following additional derivatives were prepared: for corticosterone, (a) oxidation of the corticosterone-21-acetate to 11-dehydrocorticosterone-21-acetate *via* CrO_3(8), and (b) saponification of "a" to 11-dehydrocorticosterone by means of methanolic $KHCO_3$(9); for cortisol, (a) the cortisol-21-acetate was oxidized to cortisone acetate(8), and on the 11β-HP samples adrenosterone was prepared from the cortisol(10). These derivatives were chromatographed in appropriate paper systems (7), eluted, and S.A. determined.

Results obtained upon incubation of C^{14}-steroid substrates with bovine adrenal homogenates are shown in Table I. In Ia, aliquots of a sucrose homogenate were incubated with an equal number of counts (2.4 x 10^6 c./m.) of C^{14}-11β-HP and C^{14}-DOC; results obtained with C^{14}-progesterone in identical sucrose homogenates in two other experiments are shown in Ib for comparison. It will be seen that whereas the incorporation of 11β-HP-4-C^{14} into cortisol or corticosterone could not be detected, incorporation of C^{14}-DOC into corticosterone and C^{14}-progesterone into both corticosteroids is readily demonstrable. In I, the total number of μM of C^{14}-steroid substrate incubated were different; the 11β-HP had a higher S.A. and was present in lowest concentration. In II, C^{14}-11β-HP, C^{14}-progesterone and C^{14}-DOC (all at a concentration of 2.8-2.9 μM per 100 g tissue equivalent), were incubated in various homogenates all derived from the same pool of adrenocortical brei. In this experiment, C^{14}-11β-HP was incorporated into both cortisol and corticosterone in all media. However, C^{14}-11β-HP is incorporated into the corticosterone synthesized to a lesser extent than either C^{14}-progesterone or C^{14}-DOC, independently of the type of media employed. With regard to the conversion of 11β-HP to cortisol relative to progesterone, the results differ depending upon the media used. In sucrose homogenates, wherein C^{14}-progesterone conversion to cortisol occurs at the greatest rate, progesterone is superior to 11β-HP in confirmation of the results obtained in I. When homogenates are prepared with ionic media, C^{14}-progester-

TABLE I. Products Isolated from Bovine Adrenocortical Homogenates Prepared in Various Media and Incubated with C^{14}-Substrates.

	C^{14}-substrate*	Tissue wt (wet), g	C^{14}-substrate added/100 g tissue μM	Cts/min. (× 10^6)	Medium	Net steroid production (mg/100 g/2 hr) B†	F‡	C^{14}-substrate conversion (μg/100 g/2 hr) B	F	S.A. (cts/min./μg) B	F	S.A. B / S.A. F
Ia	11β-HP-4-C^{14}	44	1.1	2.47	Sucrose	1.89	.71	<0.2	<0.2			
	DOC-21-C^{14}	"	4.6	2.36	"	2.50	.76	415	<0.5	257		
Ib	Progesterone-21-C^{14}	100	1.7	.87	"	2.12	1.52	76	27	57	28	2.0
	Progesterone-4-C^{14}	60	1.6	.80	"	2.60	2.58	123	52	76	32	2.3
II	11β-HP-4-C^{14}	35	2.8	1.47	"	2.39	.87	21	4	14	7	1.9
					KCl	.69	1.16	13	7	29	9	3.4
					NaCl	.97	.70	29	7	46	16	3.0
	Progesterone-4-C^{14}	"	2.9	1.47	Sucrose	1.31	.89	51	18	62	33	1.9
					KCl	.68	.82	34	2	80	5	17.0
					NaCl	1.18	.77	81	4	110	8	13.6
	DOC-21-C^{14}	"	2.8	1.47	Sucrose	1.98	.76	137	<0.5	107		
					KCl	1.04	1.09	45	"	67		
					NaCl	1.02	.82	72	"	110		
III	11β-HP-4-C^{14}	46	3.8	1.97	KCl	.88	.74	21	9.6	37	20	1.9
	Progesterone-21-C^{14}	"	4.1	2.08	"	2.09	1.16	182	9.5	139	13	10.7
	DOC-21-C^{14}	"	4.5	2.36	"	1.52	1.20	145	<0.5	148		

* Specific activity (S.A.), cts/min./μg: 11β-HP, in I 6580, in II and III 1550; progesterone 1600; DOC 1550.

† B = corticosterone. ‡ F = cortisol.

one conversion to cortisol is markedly reduced, whereas that of 11β-HP is little affected; the net result is that under these conditions C^{14}-11β-HP is incorporated into the cortisol formed at an equal or greater extent than C^{14}-progesterone. To check this result, in III aliquots of an homogenate prepared in KCl were incubated with C^{14}-11β-HP, C^{14}-progesterone and C^{14}-DOC. The results with regard to incorporation into the cortisol synthesized were essentially similar to those obtained in II.

Table I shows incorporation of C^{14}-steroids into corticosteroids, based upon radioactivity present following chromatography of cortisol and corticosterone as the free steroid alcohol and as the monoacetate. To determine whether the corticosteroids isolated from 11β-HP incubations were radiochemically homogeneous at this stage of purification, 50% portions of the original column eluates of the 11β-HP incubations were combined to obtain separate pools of the ionic and sucrose incubations. For comparison the eluates from C^{14}-progesterone incubations were likewise pooled. These pooled samples were chromatographed, derivatives prepared, and rechromatographed at each stage. The specific activities of these samples at various stages are shown in Table II, from which it will be seen that the cortisol and corticosterone from the sucrose incubations may be considered radiochemically homogeneous at the acetate stage. However, the homogeneity of the products of the incubations in ionic media remains questionable despite the fairly rigorous purification procedures.

Discussion. It seems clear from the S.A. data in Table II, that 11β-HP-4-C^{14} can be converted to both cortisol and corticosterone in the systems employed. The question then arises whether 11β-HP is a major intermediary in the reaction sequence, progesterone to cortisol or corticosterone. It is a biochemical maxim that in any reaction sequence a postulated intermediary reaction should proceed at

TABLE II. Specific Activities of Products Derived from C^{14}-11β-HP and Progesterone at Successive Stages of Purification.

Products derived from C^{14}-substrates	S.A. counts/min./μg			
	Sucrose incubations		Ionic incubations*	
	11β-HP	Progesterone	11β-HP	Progesterone
Cortisol†	35	39	27	23
" ‡	18	31	20	12
Cortisol-21-acetate†	10	36	11	7
Idem ‡	7	36	10	6
Cortisone-21-acetate	5	32	8	7
Adrenosterone	5		4	
Corticosterone†	49	64	154	154
" ‡	46	68	140	136
Corticosterone-21-acetate†	11	67	48	126
Idem ‡	11	53	48	132
11-dehydrocorticosterone-21-acetate	8	59	36	108
11-dehydrocorticosterone	13	55	33	99

* Products from KCl and NaCl incubations combined.

† After 1st chromatogram.

‡ " 2nd " .

a rate equal to (if not greater than) the rate of the overall process. From this point of view, it is clear that 11β-HP cannot be the major intermediary in the conversion of progesterone to corticosterone, since under all conditions studied, the conversion rates were less than those obtained with progesterone or the alternative intermediary, DOC. On the other hand, DOC is converted to corticosterone at rates equal to or exceeding those of progesterone.

The role of 11β-HP as an intermediary in cortisol production is more difficult to interpret because the incorporation of C^{14}-progesterone into cortisol is influenced by the nature of the homogenate preparation. In sucrose homogenates, where C^{14}-progesterone is incorporated into cortisol at the highest rate, C^{14}-11β-HP is definitely inferior. When ionic media are used, C^{14}-progesterone apparently does not mix well with endogenous progesterone at the site of the 17-hydroxylation step, since despite the fact that endogenous cortisol production is not diminished the incorporation of C^{14}-progesterone is markedly reduced. This is also evident from the fact that with C^{14}-progesterone the ratio of S.A.'s of corticosterone/cortisol is increased from about 2 in sucrose to 11-17 in ionic media.

To determine the significance of 11β-HP as cortisol intermediary, one must decide which type of homogenate most nearly exhibits the pattern of corticosteroidogenesis observed in the intact adrenocortical cell. Fortunately, data are available to help decide between these alternatives. In experiments wherein C^{14}-progesterone was perfused through bovine adrenals(11), it was found that the S.A. ratio of the products formed (S.A. corticosterone/S.A. cortisol), was 1.5; this is of the same order of magnitude as in the sucrose homogenates. Thus it would appear that the components involved are organized in sucrose homogenates in a manner that is similar to the arrangement in intact cells of the perfused gland, whereas the conditions in the homogenates prepared with ionic media are "abnormal" relative to intact cells. From these considerations, the conclusion emerges that 11β-HP, while capable of being converted to cortisol by adrenal enzymes, is *not* a major intermediary in the formation of cortisol from progesterone in the cow gland.

Summary. Trace amounts of C^{14}-11β-hydroxyprogesterone, C^{14}-progesterone, and C^{14}-DOC were incubated with homogenates of adrenocortical tissue prepared in various media. These homogenates, which synthesize corticoids from endogenous precursors, hydroxylated C^{14}-11β-hydroxyprogesterone at both the C-21 and C-17 positions, producing cortisol and corticosterone. However, the limited extent of these conversions relative to that of known precursors (progesterone and DOC) indicates that 11β-hydroxyprogesterone is not a major intermediary in the biosynthesis of these corticoids from progesterone.

1. Hechter, O., Zaffaroni, A., Jacobsen, R. P., Levy, H., Jeanloz, R. W., Schenker, V., Pincus, G., *Recent Progr. Hormone Research,* 1951, v6, 215.

2. Hechter, O., Pincus, G., *Physiol. Rev.,* 1954, v34, 459.

3. Levy, H., Jeanloz, R. W., Jacobsen, R. P., Hechter, O., Schenker, V., Pincus, G., *J. Biol. Chem.,*

1954, v211, 867.

4. Brownie, A. C., Grant, J. K., Davidson, D. W., *Biochem. J.*, 1954, v58, 218.

5. Saba, N., Hechter, O., *Fed. Proc.*, 1955, v14, 775.

6. Eichhorn, J., Hechter, O., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 311.

7. Zaffaroni, A., *Recent Progr. Hormone Research*, 1953, v8, 51.

8. Sarett, L. H., Johns, W. F., Beyler, R. E., Lukes, R. M., Poos, G. I., Arth, G. E., *J. Am. Chem. Soc.*, 1953, v75, 2112.

9. Reichstein, T., von Euw, J., *Helv. Chim. Acta*, 1938, v21, 1181.

10. Zaffaroni, A., Burton, R. B., *J. Biol. Chem.*, 1951, v193, 749.

11. Stone, D., Hechter, O., *Arch. Biochem. and Biophys.*, 1954, v51, 457.

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Anti-hypertensive Action of 17-Imidazolyl-steroids in the Rat. (23824)

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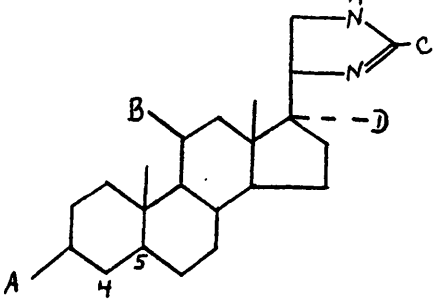
Metacorticoid hypertension is a self-sustaining cardiovascular disease attained in rats some 3 months after the implantation of a desoxycorticosterone acetate (DCA) pellet and the substitution of saline for drinking water. In many respects, this type of hypertension appears similar to essential hypertension in man(1) and responds to treatment with various drugs active in the human disease(2,3). In addition, certain steroids(4-6) and steroid-analogs(7) have been reported to exert transient depressor effects in metacorticoid hypertension. The present report describes similar activity in a new series of compounds, 17-imidazolyl-androstenes(8).

Acute experiments. This study utilized 70 male, Sprague-Dawley rats that had received subcutaneously a 40 mg wax pellet containing 20 mg DCA at least 3 months previously and that had been maintained on 0.86% NaCl solution and Rockland Rat Diet *ad libitum*. Saline and food were not removed during the course of the experiment. The systolic blood pressures of treated rats were estimated without anesthesia or heating using a photoelectric tensometer(9) before and 2, 4 and 6 hours after treatment, and were compared to simultaneous readings from propylene glycol-injected controls. Pressure readings on individual rats were made in ignorance of the injection the rat had received and of the previous pressure reading. Each test usually consisted of 4 treated and 4 control rats run simultaneously by one person on the same tensometer in a darkened room, with injections made by a different person. In order to minimize leakage, subcutaneous injections were always made at least 60 mm from the site of skin puncture with a 20 g hypodermic needle. The following 6 steroids were injected in a total of 35 rats as 1% solutions in propylene glycol at a dosage of 20 mg/kg body weight:



	A	B	C	D	Δ
SC-4200	OH	H	H	H	5
-4225	O	H	CH_3	H	4
-4577	O	H	H	OH	4
-6243	O	H	H	H	4
-6255	OH	H	CH_3	H	5
-6307	O	O	H	OH	4

Table I lists the mean pretreatment blood pressures and the changes observed after treatment with the 6 steroids; all control data are pooled. The probability values were read from Fisher's Table I(10) after applying the rank-sum test(11) to the blood pressure changes. On this basis, significant hypotensive effects were produced only by SC-4200