dilution factor inherent to ion-exchange chromatography. Molecules heavily labeled with fluorescein, which have been incriminated as a cause of NSS, may be removed by DEAE chromatography, but not by gel filtration. However, the importance of this function is diminished by recent technical advances that allow the production of conjugates possessing practically any F:P ratio desired(4).

Elimination of 19S globulin from conjugates is advantageous only when this globulin makes no important contribution to specific staining. Numerous studies have been made to determine the types of globulin with which antibody is associated. The distribution varies with the particular antigen employed, the species of animal immunized, and the intensity of immunization(12). Fractionation of conjugates by gel filtration as described in the present study should prove useful in establishing the effect of these variables upon the type of globulin in which antibody appears.

Summary. Gel filtration proved to be a simple, effective method for separation of fluorescein-labeled antiglobulin for *E. coli* into 7S and 19S fractions. Both fractions were found to contribute considerable nonspecific staining, while specific staining was associated predominantly with the 7S globulin. The ratio of specific staining titer to nonspecific staining activity was 3-fold greater with a pool of fractions in which only 7S globulin was detected than with the unfractionated conjugate when the 2 preparations were tested at the same protein concentration.

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Metabolism of Labelled Steroid Precursors by Normal Bovine Adrenal Medulla *in vitro*.* (30291)

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Experimental evidence has recently been adduced supporting the view that chromaffin tissue possesses to a significant degree the enzymatic actions of the adrenal cortex at several stages of the currently accepted scheme of steroid biosynthesis(1,2). This suggestion was put forward as a result of our studies with chromaffin tumors incubated with various radioactive precursors. The 3 major steroid hydroxylases (21, 11β and 17α), the

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catalytic agent responsible for C_{17}-C_{20} side-chain scission and Δ^5,3β-hydroxysteroid dehydrogenase were present in the pheochromocytomas. The tumors failed to degrade cholesterol, denoting lack of the "desmolase" required to effect side-chain cleavage and/or absence of the hydroxylases (*20α,22*) acting prior to this step.

It was thought of interest, therefore, to test the effectiveness of normal chromaffin parenchyma in this regard and also to extend our survey to earlier precursors lacking a cyclic configuration. This report concerns the outcome of our *in vitro* investigations with normal bovine medullary tissue, using the cortex as a control. The comparison will be limited mostly to the over-all transformation of substrates. The spectrum of reaction products, their individual quantitation and radiochemical characterization will be reported in detail later.

Materials and methods. Fresh bovine adrenal glands obtained at the local abattoir were transported to the laboratory in ice-cold Krebs-Ringer solution. A number of glands, far exceeding our experimental needs, was always procured, so as to allow a preliminary screening of specimens. Only those showing a definite demarcation between cortex and medulla were further processed. Following removal of surrounding fat, the glands were sliced transversely along a plane perpendicular to the longest axis, the slices containing islets or invaginations of the cortex into the medulla being discarded. In the selected sections, a peripheral rim, consisting of the whole cortex and the contiguous area of the medulla, was separated, the adjacent chromaffin tissue being later carefully removed and discarded. The pure cortical rim (dark brown) was then cut very finely.

The central portion of the medulla (yellowish pink) was then processed under a magnifying glass and denuded of brownish zones and visible vasculature. Only after this meticulous dissection was the medullary preparation subjected to fine chopping. Specimens were taken for histological studies and the rest of the tissues were twice thoroughly washed, blotted and weighed in aliquots of 1 g, (unless otherwise specified). All these manipulations were performed maintaining the tissues cold and lasted for less than 3 hours.

When surviving slices were used, the 1 g aliquots were transferred into Pyrex vessels containing 10 ml of Krebs-Ringer-bicarbonate-glucose solution, in which the radioactive precursors had previously been dissolved.†

Identical vessels were also prepared in which the basic medium was supplemented with NAD or NADP (0.6 μmole/ml of either) and G-6-P (1.8 μmole/ml) just before incubation started. The efficiency of NADP + G-6-P to generate NADPH indicates the existence of G-6-P-dehydrogenase in both types of tissues. An approximate ratio of 1:6 (medulla = 325; cortex = 1800 units/g) of this dehydrogenase has been reported by Kelly *et al*(3) for beef adrenals. Other additives (ACTH, catecholamines) will be considered under *Results*. Incubation was carried out for 3 hours. This final incubation was preceded by a 1-hour preincubation period of all flasks when ACTH or pressor amines were used.

In studies conducted with homogenates, the tissues were macerated in tightly fitting all-glass homogenizers, using phosphate-buffered Krebs-Ringer solution prepared with 0.04 M fumarate and fortified with 0.04 M nicotinamide. Following filtration through gauze, adequate aliquots were pipetted into flasks containing the dissolved radioactive precursors. The system was then supplemented with NADP and G-6-P at the same concentrations stated for slices and the final volume was 10 ml. In selected cases NADPH, ATP and NAD were mixed in the homogenates prior to incubation, instead of the NADPH-producing mixture (Table II).

† Sources of organic compounds: All ^{14}C-labelled precursors were obtained from New England Nuclear Corp. NAD (nicotinamide adenine dinucleotide), NADP (nicotinamide adenine dinucleotide, phosphate, monosodium salt), NADPH) reduced form of previous coenzyme, tetrasodium salt), ATP (adenosine triphosphate, disodium salt), G-6-P (glucose 6-phosphate, disodium salt) L-epinephrine (bitartrate) and L-norepinephrine (bitartrate) from Sigma Chem. Co. Dopamine (3-hydroxytyramine HCl) from Calbiochem. ACTH (Corticotropin) from Nordic Biochem. Ltd. Lot A-3601.

TABLE I. Conversion of Labelled Acetate by Cortical and Medullary Preparations.

Substrate	Experimental conditions	Total conversion, cpm × 10^4 Cortex	Medulla
Acetate-1-^{14}C, 166 μc	Slices:		
	No additions	1.2 (.006)*	1.1 (.005)
	ACTH (10 i.u.)	5.3 (.024)	1.0 (.004)
	NADP + G-6-P	1.3 (.006)	.5 (.002)

* Figures in parentheses denote % conversion. One hr preincubation in all experiments.

Radioactive materials serving as substrates and their varying amounts, will be indicated under *Results*. Their purity was verified before being employed by chromatographing 2-4 μc. In some experiments stable compounds were mixed with the corresponding isotopically labelled substrates in amounts ranging from 15-20 μg/ml of medium, with the idea of stockpiling enough steroidal substances for identification purposes.

Other conditions of incubation, methods for extraction (freshly distilled ethyl acetate and chloroform, 1:1) and preparation of neutral extracts, have been performed essentially as previously reported(2). The same reference gives a detailed account of the paper chromatography serial fractionation scheme used for resolution of steroids and the procedures for detection and measurement of radioactivity.

Results and discussion. Acetate. When sodium acetate-1-^{14}C was used as substrate at the level of 25 μc, slices of cortex and medulla failed to give rise to discernible conversion products, even after addition of ACTH, up to 60 i.u. Increasing the concentration of acetate (166 μc), slices of both tissues showed an equal capacity to incorporate the 2-carbon substrate into polar C_{21} steroids. Ten i.u. of ACTH produced a 5-fold increase in the transformation by the cortex, whereas it failed to stimulate the medulla. No enhancement was observed in either case by a NADPH-generating system; actually, a marked decrease was observed with the medulla (Table I). Similar responses to our control experiments have recently been reported for the adrenal cortex(4). The transformation occurring in the medulla is not ACTH-dependent and seemingly unrelated to any cortical remnants. Cortisol accounted for most of the conversion by the cortex; corticosterone by the medulla. In either case, the search for early intermediates, *i.e.*, cholesterol, was unsuccessful.

Mevalonate. Radioactive zones with the chromatographic mobility of cortisol (toluene/ethylene glycol system, 48 hours) were encountered in cortical and medullary incubates (slices and homogenates) employing as substrate mevalonic-2-^{14}C (25 μc of the dibenzylethylenediamine salt). Further chromatography after addition of cold carrier and following oxidation with CrO_3 (Bush B_5 system) revealed the non-identity of these labelled materials to cortisol or any other known adrenal steroid. When 25 μc of substrate were extracted without incubation with tissues, the same band appeared as a contaminant of mevalonic acid detectable only when this higher amount of material was tested. No indication of cholesterol synthesis was obtained with any type of tissue preparation. These failures are in agreement with the results previously reported by Bryson and Sweat(5) using bovine cortical homogenates and which led the authors to question the role of mevalonic acid as a precursor of corticosteroids.

Cholesterol. Curiously, we have encountered serious difficulties in the *in vitro* utilization of cholesterol-4-^{14}C (2-4 μc) by cortical slices under a variety of conditions of experimentation, including additions of ACTH, ATP and 3′,5′-adenosine monophosphate (unpublished data). Only by supplementing the medium with a NADPH-generating system have we succeeded, with slices, in obtaining incorporation of the C_{27} sterol into corticosteroids. Table II indicates that the degree of conversion bears a direct relationship to the amount of cortical tissue. Chromaffin preparations invariably failed to reproduce these results.

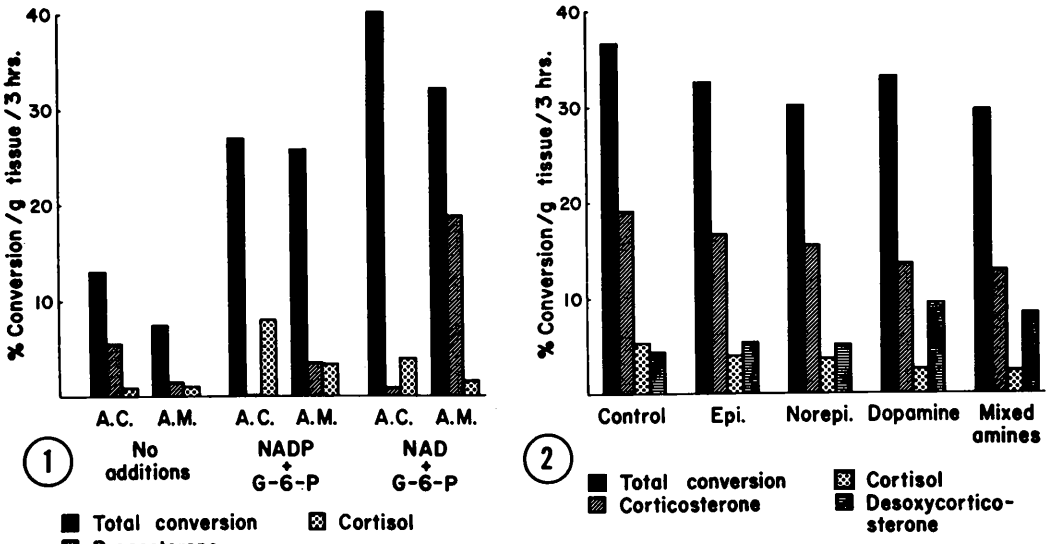

FIG. 1. Conversion of Δ^5-pregnenolone-4-^{14}C (1 μc) by medullary (A.M.) and cortical (A.C.) slices of beef adrenals. Steroids accounting for total conversion are α-ketols with a Δ^4,3-keto grouping (mainly corticosterone, cortisol and desoxycorticosterone) and progesterone (when present). No preincubation period.

FIG. 2. Effects of catecholamines on steroid biosynthesis by bovine adrenal cortical slices. Steroid precursor: Progesterone-4-^{14}C (2 μc). Epi.: epinephrine, Norepi.: norepinephrine, Dopamine: 3-hydroxytyramine, Mixed Amines: Epi. + Norepi. + Dopamine. Concentration of each amine: 200 μg/ml of buffer solution. One hour preincubation.

In contrast to slices, cortical homogenates, representing as little as 25 mg of parenchyma, easily catabolized cholesterol-4-^{14}C (2 μc) to cortisol, most of the expected intermediates being detectable. Increasing the tissue concentration enhanced the degree of over-all transformation and the appearance of metabolites. Efforts to duplicate this sequence with medullary homogenates consistently failed. No conversion products were demonstrable using up to an equivalent of 2.5 g of medulla (Table II) or by raising the amount of substrate to 20 μc. These results demonstrate a complete divergence in the metabolic actions of cortex and medulla at a step regarded as rate-limiting in steroidogenesis(6) and indicate that cortical contamination in our medullary preparation is less than 1%. Identical findings were obtained with phenochromocytomas (4 g), where it was demonstrated in addition that neither the catecholamines nor the presence of chromaffin cells alter the capacity of cortical homogenates for side-chain splitting of cholesterol(2).

Pregnenolone. Fig. 1 depicts the ability of cortical and medullary slices to convert Δ^5-pregnenolone-4-^{14}C (1 μc) under various experimental conditions. Without exogenous coenzymes, the medulla exhibited more than half of the capacity of the cortex for utilization of the precursor. Considerable augmentation of reaction products was attained by either tissue under the influence of NAD or NADP supplemented with G-6-P. The in-

TABLE II. Percentage Conversion of Cholesterol-4-^{14}C (2 μc).

Tissue preparation (wt in mg)		Cortex	Medulla
Slices:*	125	.3	0
	250	.7	0
	500	1.2	0
Homogenates:†	25	3.1	0
	50	4.3	0
	100	6.3	0
	1000	11.7	0
	2500	15.4	0

* Medium supplemented with a NADPH-generating system.

† These homogenates were incubated with NADPH, ATP and NAD. The concentration was 0.33, 2 and 0.5 μmole/ml of homogenate respectively (total vol 10 ml). Similar results were obtained by replacing these additives by NADP + G-6-P at concentrations given in *Methods.*

crement over control values was greater in the case of the medulla with either cofactor (NADP: cortex = + 109%; medulla = + 248%. NAD: cortex = + 207%; medulla = + 333%.)

It is of interest that with either type of tissue, labelled α-ketols were obtained with NAD, a coenzyme which in adrenal cell-free preparations promotes transformation of pregnenolone only as far as progesterone(7,8). The occurrence of subsequent steroid hydroxylations may be explained in slices, on the basis of an accelerated generation of NADPH from endogenous sources in the presence of added G-6-P. A salient feature of these experiments with NAD, however, is the remarkable accumulation of progesterone (18.7%) achieved by the medulla in contrast to the cortex (0.82%). These results not only provide unequivocal evidence of the presence in chromaffin cells of the NAD-linked $\Delta^5,3\beta$-hydroxysteroid dehydrogenase, but strongly suggest different modes of utilization of pregnenolone by the two tissues. Although in lesser quantities (3.4%), progesterone was also encountered when medullary incubates were supplemented with a NADPH-generating system. Under these circumstances the conversion products from cortical slices were exclusively α-ketols, reproducing the results obtained by Daily *et al* (8) with hog adrenal homogenates, in which no progesterone was detectable after addition of NADPH.

Progesterone. The total transformation of progesterone-4-^{14}C (4 μc) produced by medullary slices was 30-40% of that of the cortex. Fluctuations in the rate of utilization of progesterone occurred with different glands but this 1:3 ratio remained quite constant. Additions of NADP + G-6-P markedly increased the enzymatic capacity of either tissue. In a representative experiment these additives enhanced the conversion by the cortex from 18.4 to 55.2% (+ 200%) and by the medulla from 6.9% to 36.8% (+ 434%), denoting a greater responsiveness by chromaffin cells. Both parenchymas, especially in the presence of the electron donor, produced no less than 15 well individualized steroids, the large majority as a result of insertion of oxygen atoms at different sites of the substrate. Cortisol and corticosterone predominated as reaction products in both instances.

Homogenates metabolized progesterone to a greater extent and more consistently. In incubation studies with 4 μc of radioprogesterone and increasing the amounts of either tissue, homogenates equivalent to 1 g of medulla exhibited the same hydroxylating capacity as 300 mg of cortical homogenates (30%). Ten mg of cortical homogenates (representative of a 1% contamination) showed less than 5% of the effectiveness of 1 g of medullary homogenates.

Epinephrine, norepinephrine and dopamine, when added individually or combined, did not increase the total conversion of progesterone attained by slices of the cortex (2 μc, Fig. 2) or by homogenates (4 μc). The catecholamines, however, increased the concentration of desoxycorticosterone in the medium, seemingly at the expense of 11β- and 17α-hydroxylated compounds. Fig. 2 shows that these changes were very slight with epinephrine and norepinephrine but quite pronounced with dopamine. We have not been able to corroborate the previous claim that pressor amines stimulate the hydroxylating capacity of cortical homogenates(9).

17α-Hydroxyprogesterone and 11-desoxycortisol. These ^{14}C-labelled substrates (0.5 μc, each) were used primarily to test the side-chain splitting capacity of either tissue from a C_{21} substrate. In this instance, instead of the whole cortex the zona fasicculata-reticularis served as control. At the substrate level used, no $C_{21} \rightarrow C_{19}$ conversion occurred with the inner cortex. With the medulla, small bands with the chromatographic mobilities of androstenedione (0.4%) and 11β-hydroxyandrostenedione (0.9%) were isolated from the experiment with 17α-hydroxyprogesterone; only androstenedione (0.4%) from 11-desoxycortisol. It seems of interest to report these experiments here since NADPH while practically not affecting the conversion of these substrates to cortisol by the inner cortex, once again produced a tremendous stimulation in the case of medullary incubates (+ 491% for 17α-hydroxyprogesterone and + 1435% for 11-desoxycortisol). The co-

enzyme, however, did not produce changes in conversion to C_{19} compounds.

From the aforementioned results it is evident that the data obtained with the pheochromocytomas have been reproduced with normal bovine medulla. Additional information has been gained by the cyclization of acetate by normal chromaffin cells. This action is surprisingly of the same order of magnitude in both components of the adrenal. Since the utilization of cholesterol remains restricted to cortical tissue, the transformation of acetate to corticosteroids by the medulla naturally leads to the assumption of a different metabolic pathway in this tissue, involving a complete and obligatory detour bypassing cholesterol.

An interesting phenomenon is the remarkable sensitivity of chromaffin cells to exogenous coenzymes, which is magnified by increasing the degree of oxygenation of the precursor. That it might reflect different species of "mixed-function" steroid oxidases seems an attractive speculation.

The issue of cortical contamination, discussed at length elsewhere(2), will be reexamined briefly here. Histological studies revealed a minute cortical infiltration in some of our preparations. The transformation achieved from several of the precursors however seems grossly out of proportion to the number of cortical cells. The experiments using progesterone with different amounts of cortex and medulla support this view. That these results are not due to contamination is borne out by our finding of a distinctly different metabolic behaviour of the two tissues in various experiments. In this respect, the following medullary effects should be recalled: accumulation of progesterone from pregnenolone, failure to increase corticoid formation from acetate in response to ACTH and above all, the inability to effect the NADPH-dependent(7) side-chain cleavage of cholesterol, in spite of the higher responsiveness of medullary preparations to additions of this coenzyme in other respects. Stimulation of some cortical remnants by the endogenous catecholamines appears equally untenable.

Summary. 1) Normal bovine adrenal medulla can effect the following reactions *in vitro*: a) Transformation of a straight-chain precursor (acetate) into steroid tetracyclic structures; b) Double bond shifting from Δ^5 to Δ^4 with concomitant oxidation at C_3; c) Introduction of -OH groups at specific sites of the pregnane nucleus and d) Conversion of C_{21} to C_{19} steroids. 2) It has consistently failed to metabolize cholesterol to corticosteroids. 3) It has shown in several steroid biosynthetic reactions a greater responsiveness to diphospho- and triphosphopyridine nucleotides than the cortex.

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