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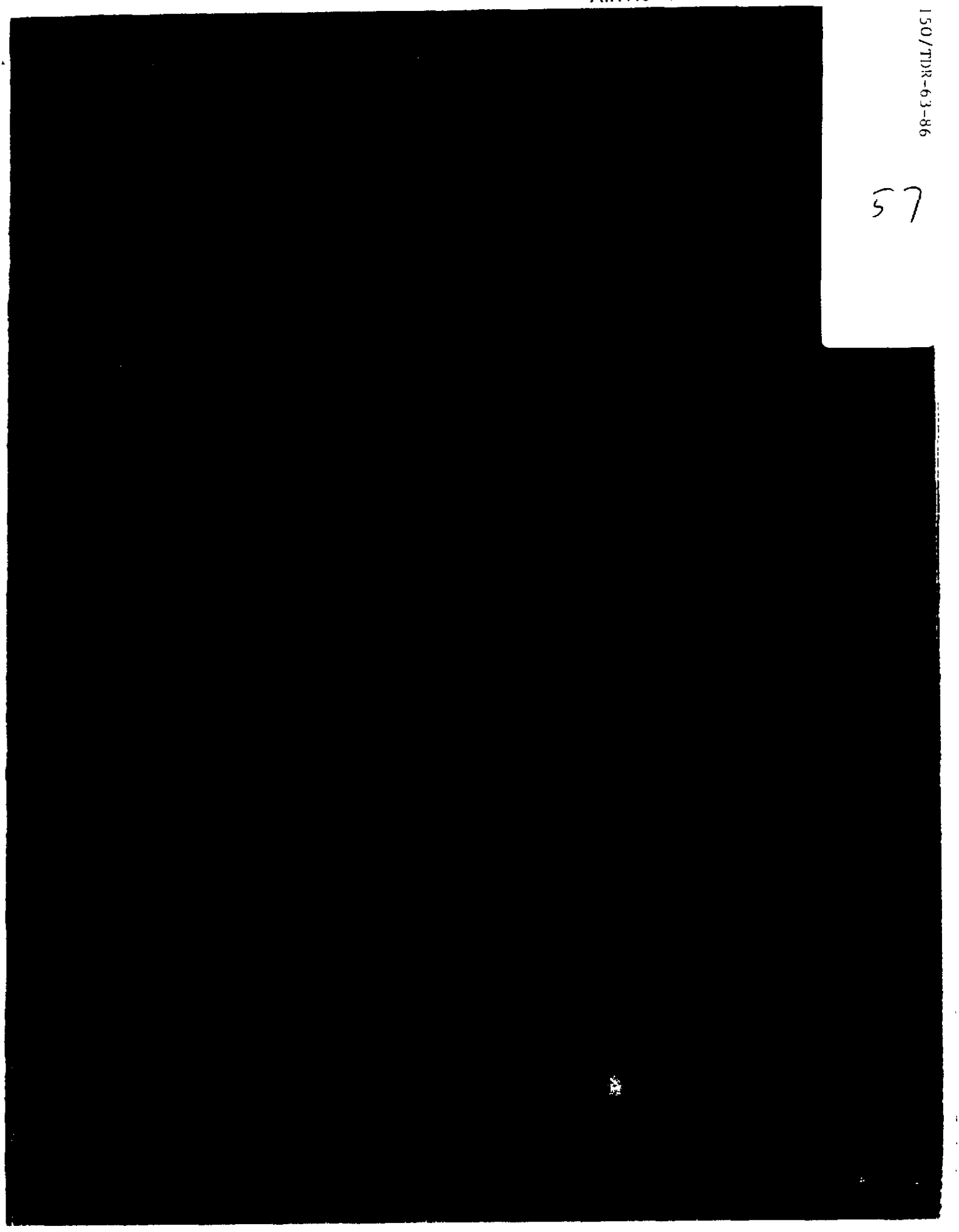

FOREWORD

This report was prepared by the following personnel:

FRED H. KATZ, Captain, USAF, MC*

IRA L. SHANNON, Major, USAF, DC†

(*Department of Internal Medicine, †Dental Sciences Division)

Patients at the Wilford Hall USAF Hospital were studied through the courtesy of Dr. Frank R. Lecocq and Dr. Steven C. Beering, and at the Brooke Army Hospital through the aid of Dr. Woodrow L. Pickhardt, Dr. William G. Mentzer, Dr. James K. Hedges, and Dr. Ralph S. Goldsmith.

Technical assistance was tendered by J. H. Perry, J. F. McAnear, E. D. Dinger, R. R. Hogue, G. R. Guenzler, and C. D. Steward.

Among the radioactive compounds used in this study, d-aldosterone-1,2-H^3 and 1,2-H^3-cortisol were obtained from the Endocrinology Study Section, National Institutes of Health, Bethesda, Md. Later these materials—along with carbon-14, tritium-labeled acetic anhydride, and 4-C^{14}-cortisol—were obtained from the New England Nuclear Corp., Boston, Mass. The 4-C^{14}-4-androstenedione and estradiol-17β-16-C^{14} were obtained from Dr. Joseph W. Goldzieher, Southwest Foundation for Research and Education, San Antonio, Tex.

The Autoscanner (model 880) is made by the Vanguard Instrument Co., La Grange, Ill., and the liquid scintillation spectrometer (model 725) is made by the Nuclear Chicago Corp., Des Plaines, Ill.

ABSTRACT

Parotid fluid, which can be easily collected in continuous fashion, was previously shown to possess free 17-hydroxycorticosteroid (17-OH-CS) levels which paralleled those in serum and reached a maximum two hours after corticotropin or cortisol administration to normal men. The present study demonstrated that intravenously administered cortisol rapidly appeared in the parotid fluid, and that, thus, parotid fluid 17-OH-CS levels would serve as reliable indicators of adrenal function. This was borne out by studies in a patient with Cushing's syndrome as well as in those showing adrenal hyporesponsiveness. Despite the large rise in plasma 17-OH-CS in the third trimester of pregnancy there was only a small, though significant, rise in parotid fluid 17-OH-CS. These data are compatible with the hypothesis that only nonprotein-bound free 17-OH-CS reach the parotid fluid

Conjugated 17-OHCS were not found in appreciable quantity in parotid fluid. Chemical and radioisotopic technics indicated cortisol and cortisone to be the major human parotid fluid 17-OH-CS. Parotid tissue from the dog converted cortisol to cortisone. Radioactive aldosterone, estrogen, and androgen appeared in parotid fluid after intravenous injection.

This technical documentary report has been reviewed and is approved.

Robert B. Payne

ROBERT B. PAYNE
Colonel, USAF, MSC
Chief, Operations Division

IDENTIFICATION AND SIGNIFICANCE OF PAROTID FLUID CORTICOSTEROIDS

1. INTRODUCTION

Parotid fluid is a biologic material which lends itself admirably to laboratory analysis because it can be obtained easily and continuously over many hours without the trauma or blood loss of venipuncture. In addition, it is sterile, easy to store, and low in protein content (rarely over 400 mg. per 100 ml.) (1). Previous reports described the excellent parallelism of parotid fluid free 17-hydroxycorticosteroid (17-OH-CS) level changes to those in blood following intramuscular or intravenous corticotropin (2, 3) or oral corticosteroid (4) administration to normal human subjects. In each case, with sampling performed at two-hour intervals for six hours, the maximum average levels occurred two hours after the hormone was given.

The purpose of the present study was to explore further the appearance time of plasma 17-OH-CS in parotid fluid, to apply these methods to altered states of corticosteroid function, to identify the corticosteroids in parotid fluid, and to investigate the physiologic nature of the steroids that appear in parotid fluid.

2. MATERIALS AND METHODS

Experimental subjects

The normal subjects were young men, aged 17 to 22 years, who had recently passed a military medical examination and whose environmental conditions were essentially identical. Hospitalized cases and outpatients were studied at Wilford Hall USAF Hospital, Lackland AFB, Tex., and Brooke Army Medical Center, Fort Sam Houston, Tex.

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Parotid fluid

This was obtained with sugared chewing gum stimulation by the use of a double lumen metal cap (5) whose inner lumen rests over the orifice of Stensen's duct and whose outer lumen is attached to the buccal mucosa by the use of simple bulb suction. The fluid is led to the collecting vessel by a Tygon tube connected to the inner chamber.

Pharmaceuticals

For intravenous use lyophilized corticotropin was employed and for intramuscular administration corticotropin gel was utilized. Cortisol[1] hemisuccinate was given intravenously and intramuscularly. Cortisol tablets (20 mg.) were given by mouth.

Radioactive compounds

Carbon-14 and tritium-labeled acetic anhydride and 4-C^{14}-cortisol were obtained commercially. d-Aldosterone-1, 2-H^3 was first a gift and later obtained commercially. The aldosterone was repurified by chromatography in the Bush B5 (6) and E_2B systems (7) prior to use. 1, 2-H^3-cortisol was also a gift as were 4-C^{14}-4-androstenedione and estradiol-17β-16-C^{14}. Radioactive steroids were dissolved in 0.6 to 3.0 ml. of ethanol and administered intravenously in 30 ml. isotonic saline solution.

Chemical methods and incubation technics

Plasma, serum, and parotid fluid 17-OH-CS were measured by the technic of Peterson

Steroids referred to are: aldosterone = 18-formyl-11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol = 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisone = 17α,21-dihydroxy-4-pregnene-3,11,20-trione; estrone = 3-hydroxy-estra-1:3:5(10)-triene-17-one; estradiol = estra-1:3:5(10)-triene-3:17β-diol; 4-androstenedione = androst-4-ene-3,17-dione.

et al. (8). Other chemical methods and technics of metabolic incubation have been previously described (9, 10).

Radioisotope detection

Chromatogram scanning was performed with a model 880 Autoscanner. A model 725 liquid scintillation spectrometer was used for quantitative counting. When samples were labeled with both C^{14} and H^3, balance point voltages were found for each isotope. Tritium could be essentially excluded from the carbon channel. Quenching was determined by the "channels ratio" method (11) and was constant for samples eluted from paper chromatograms. All counting was carried on to 1% accuracy.

3. RESULTS

ACTH and cortisol administration to normal men

Following injection of ACTH, either intravenously or intramuscularly, mean parotid fluid 17-OH-CS values rose steadily to a maximum at the end of the two-hour period of observation (fig. 1).

The results for cortisol are depicted in figure 2. When the cortisol was injected intravenously, the mean parotid fluid 17-OH-CS level was highest in the sample collected 0 to 20 minutes after the injection. After intramuscular administration, the peak value occurred in the 20- to 40-minute sample, and after oral ingestion the maximum level was seen at about 100 minutes.

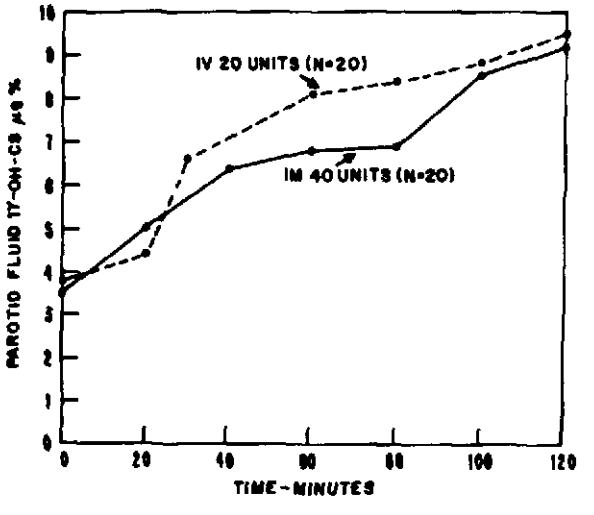

FIGURE 1

Mean values for 17-OH-CS in parotid fluid after administration of ACTH.

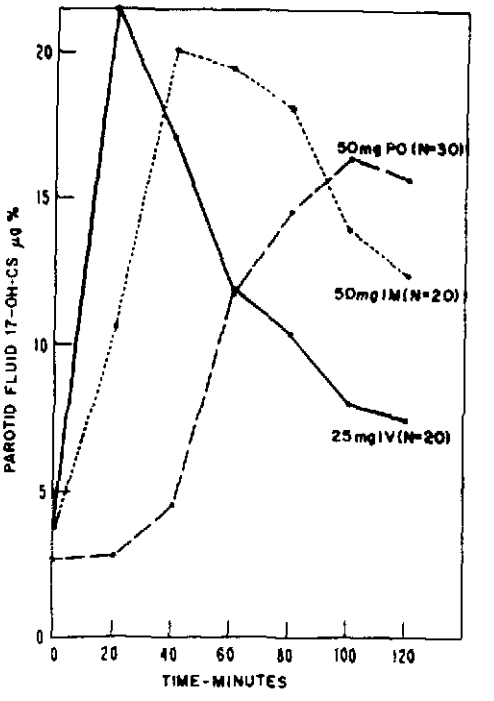

FIGURE 2

Mean values for 17-OH-CS in parotid fluid after administration of cortisol.

Abnormal responsiveness to ACTH

A 54-year-old woman with classical Cushing's syndrome, shown at surgical exploration to be associated with bilateral adrenal hyperplasia, was given 40 units of ACTH intramuscularly. It can be seen in figure 3 that her parotid fluid 17-OH-CS levels rose to 22 µg. per 100 ml. in one hour and 27 µg. per 100 ml. after two hours—values far greater than found for any of 20 normal males treated similarly.

Parotid fluid 17-OH-CS were also examined in 2 individuals with adrenal hyporesponsiveness to ACTH, as judged by plasma 17-OH-CS response to a standard infusion test (12). The upper section of figure 4 illustrates their

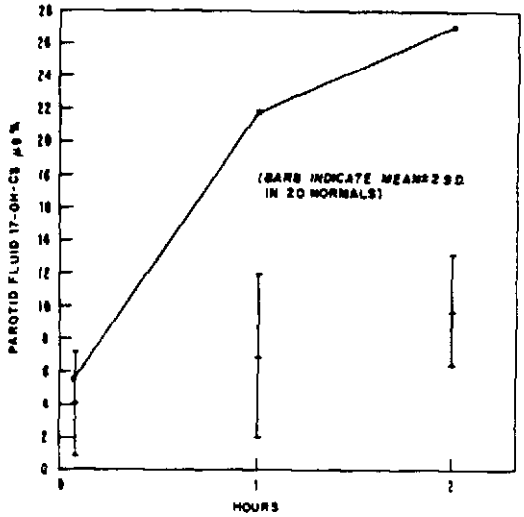

FIGURE 3

Parotid fluid 17-OH-CS values after administration of 40 units of ACTH to patient exhibiting Cushing's syndrome secondary to bilateral adrenal hyperplasia.

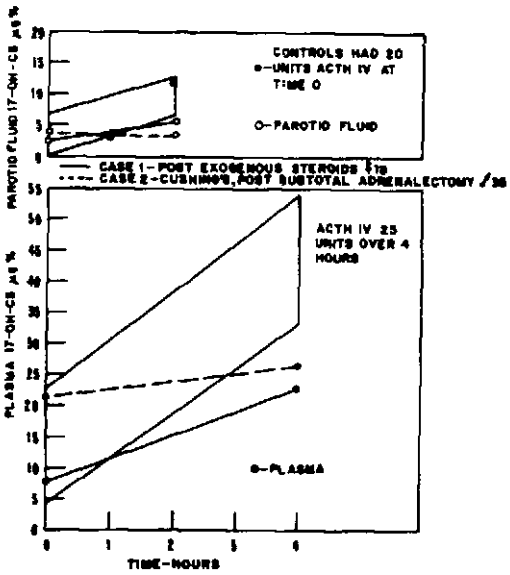

FIGURE 4

Hyporesponsiveness (in 2 patients) to intravenously administered ACTH measured simultaneously by plasma and parotid fluid 17-OH-CS.

parotid 17-OH-CS response at one and two hours after the start of the infusion. Plasma responses are seen in the lower portion of the figure. In each section the normal range is indicated by the parallelogram.

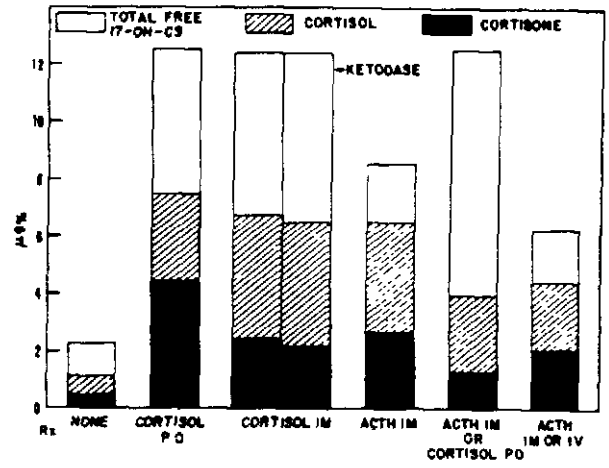

FIGURE 5

Fractionated 17-OH-CS in pools of parotid fluid.

Steroid identification

Pools containing 500 to 2,000 ml. of parotid fluid collected from normal individuals both before and after hormone treatment were extracted with ethyl acetate and chromatographed on paper in the Bush B5 and Eberlein-Bongiovanni E_2B systems prior to quantitation. In all cases, substances with the mobility of cortisone and cortisol were the only Porter-Silber positive compounds found among the reducing substances (blue tetrazolium) on the chromatograms. Figure 5 demonstrates that these two steroids (after correction for losses of 20% after each chromatogram) generally comprised over half of the total free 17-OH-CS measured in the native fluid. When the original fluid was incubated with sulfatase or beta glucuronidase, there was no appreciable additional yield of Porter-Silber positive material, as illustrated in the column marked "Ketodase."

Cortisol and cortisone isolated from parotid fluid were further identified by ultraviolet absorption, the soda fluorescence reaction, monoacetate and 17-ketosteroid derivative formation and chromatographic mobility in the Bush B3 and B1 systems, as well as spectral peaks in concentrated sulfuric acid (240 mμ for cortisol and 280 mμ for both compounds).

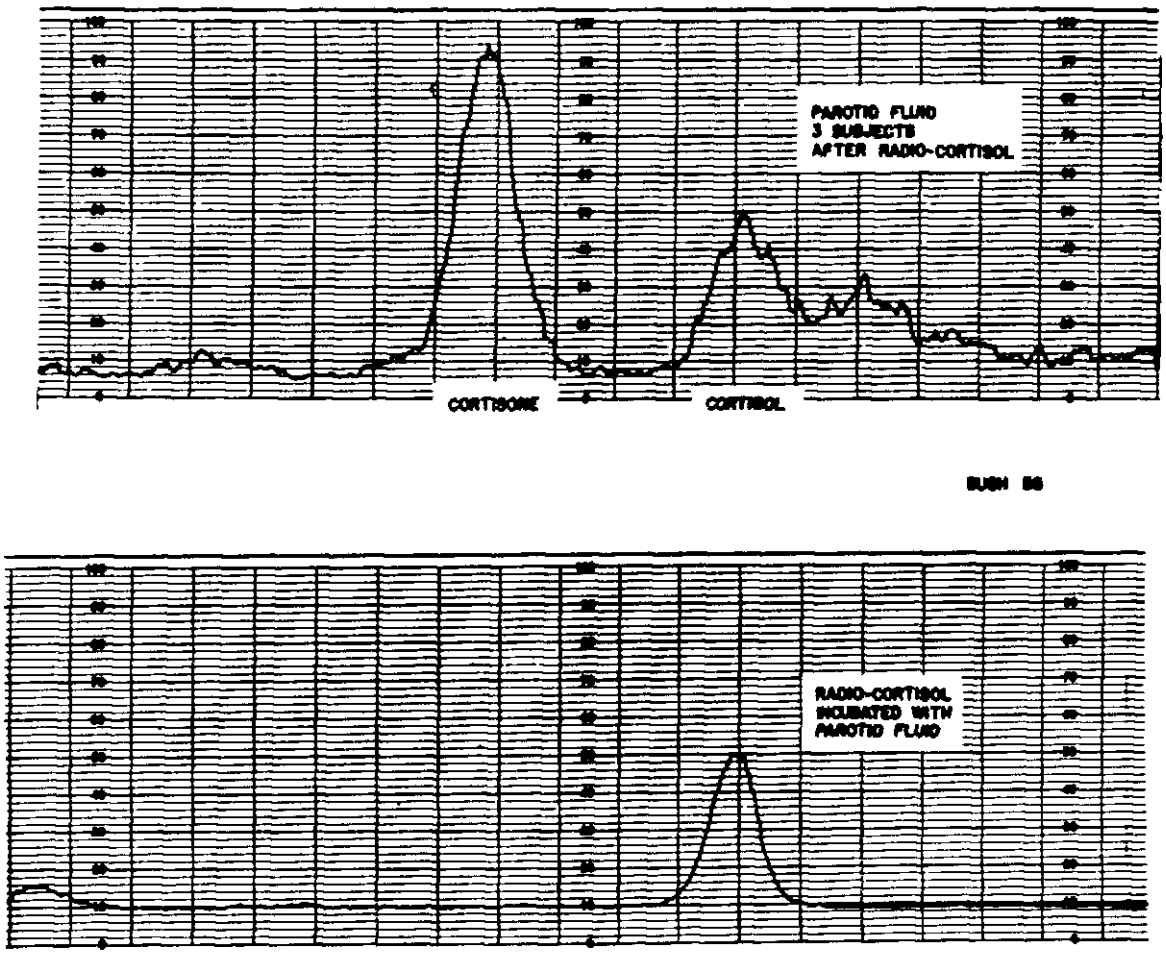

FIGURE 6

Chromatograms of ethyl acetate extracts of parotid fluid scanned for radioactivity. Stained strip of steroid standards is shown in center.

In addition, 8 normal males were given 3 μc. 4-C^{14}-cortisol intravenously, and parotid fluid, collected over the subsequent three hours from one gland, was chromatographed.

The radioactivity scan of the chromatogram in the top of figure 6 shows that the highest peaks in one pool from 3 subjects corresponded to cortisol and cortisone. The bottom scan is of a chromatogram of parotid fluid incubated with radioactive cortisol in 5% CO_2 in oxygen. A cortisone peak and a cortisol peak (from different pools) were eluted and acetylated with tritium-labeled acetic anhydride. The resulting monoacetates were repeatedly chromatographed and H^3/C^{14} ratios of the monoacetates were determined (see table I) to establish radiochemical purity.

Chromatograms of methylene chloride extracts of plasma obtained 20, 40, and 60 minutes after administration of 50 mg. cortisol intravenously to normal men failed to show the presence of any substantial amounts of cortisone, despite the high cortisol levels.

Tissue incubations

Slices of fresh dog parotid gland, incubated with cortisol-1, 2-H^3 in the presence of triphosphopyridine nucleotide appeared to hasten transformation to cortisone-1, 2-H when

TABLE I

H^3/C^{14} ratios of steroid acetates on successive chromatograms

Steroid acetates	Source of steroid	B3 system (ratio 1)	B1 system (ratio 2)	B3 system (ratio 3)
Cortisol acetate	Parotid fluid, 3 men after 4-C^{14} cortisol	2.66	2.79	
Cortisone acetate	Parotid fluid, 5 men after 4-C^{14} cortisol	1.95	1.53	1.74
Cortisone acetate	Dog parotid incubation with cortisol-H^3	.198	.198	
Cortisone acetate	Dog muscle incubation with cortisol-H^3	.085	.090	

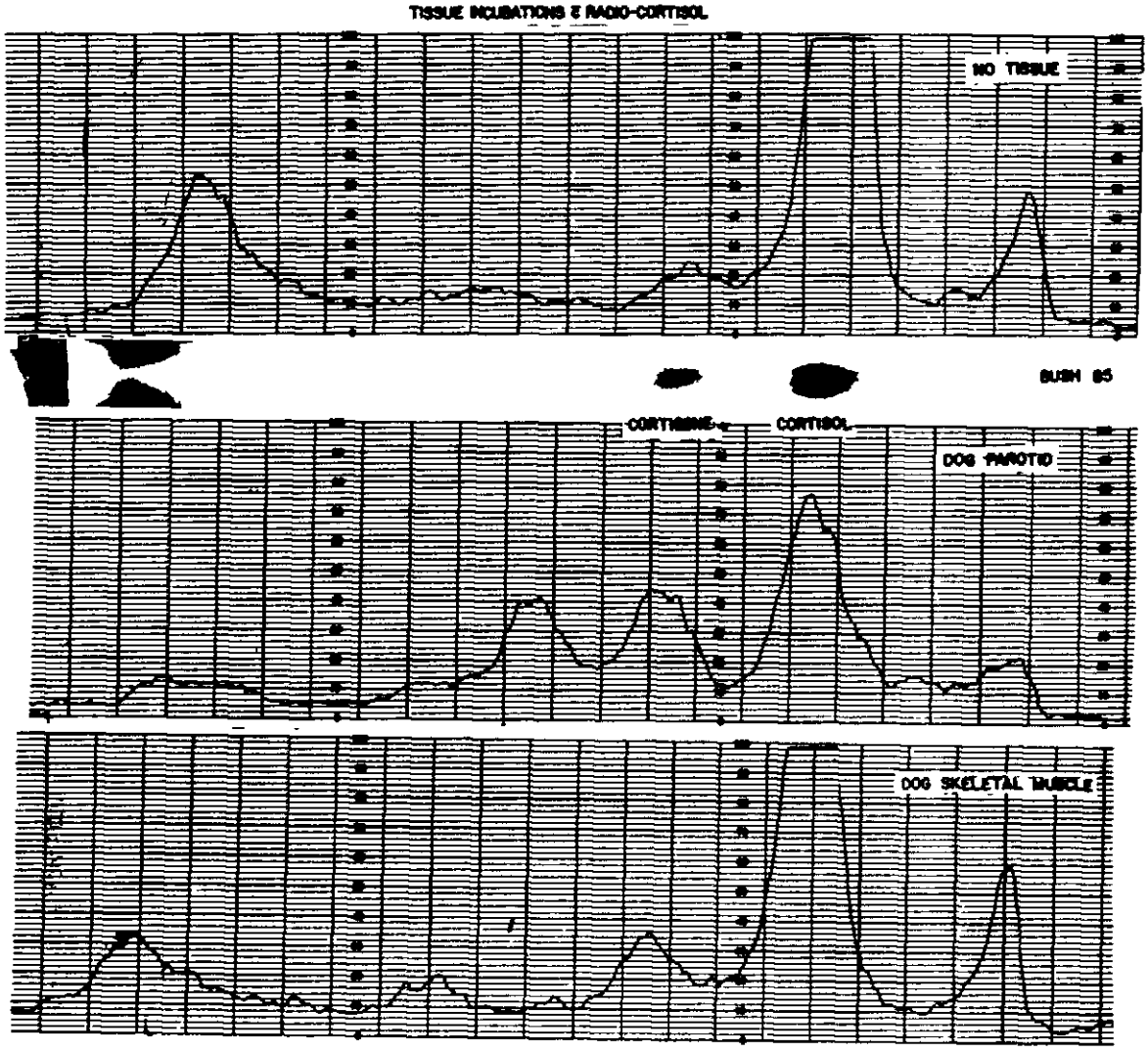

FIGURE 7

Transformation of cortisol to cortisone by animal tissues.

TABLE II

Effect of pregnancy on plasma and parotid fluid 17-hydroxycorticosteroids

	Nonpregnant controls	Third trimester
Number of subjects	20	26
Plasma 17-OH-CS μg./100 ml.		
Mean	10.6	29.1
S.D.	5.0	11.0
Parotid fluid 17-OH-CS μg./100 ml.		
Mean*	3.0	4.2
S.D.	1.6	1.6

*Difference between these means significant at the .02 level.

compared to incubation cups containing skeletal muscle or no tissue (see radioactivity scan, fig. 7). The apparent cortisone peak in the "no tissue" scan did not migrate as cortisone in the E_2B system. H^3/C^{14} ratios of the cortisone produced by parotid and muscle tissue after addition of 10 μg. carrier cortisone and acetylation with C^{14}-labeled acetic anhydride are also shown in table I.

Studies in pregnancy

Plasma and parotid fluid 17-OH-CS levels of 26 women in the third trimester of uneventful pregnancy and 20 women of similar age but six weeks postpartum, were determined between 8 and 10 a.m. and are summarized in table II. In the presence of the marked elevation of plasma 17-OH-CS, there was also a significant increase ($P < .02$) in parotid fluid 17-OH-CS levels in the pregnant subjects.

Other steroids

Three μc. of aldosterone-1, 2-H^3 were given intravenously to each of 9 normal male subjects, and chloroform extracts of the pooled parotid fluid collected over the next three hours were chromatographed. It was found that 0.025% of the original dose migrated with authentic aldosterone in the B5 and E_2B systems. Hydrolysis at pH 1 prior to extraction did not increase the yield of radioactivity significantly.

Following an injection of 800,000 counts per minute (c.p.m.) of 4-C^{14}-Δ^4-androstenedione, an ether extract (13) of an enzyme hydrolyzed-solvolyzed three-hour parotid fluid collection contained 125 c.p.m., or 0.015% of the dose.

Only 0.0001% of a dose of 5×10^6 c.p.m. of injected estradiol-17β-16-C^{14} was found in a similar parotid fluid collection. All the counts were in the estrone and estradiol fraction (14).

4. DISCUSSION

It seemed likely from the previous studies on parotid fluid 17-OH-CS following corticotropin and corticoid administration, and their lack of variation with flow rate, that levels of parotid fluid 17-OH-CS could serve as an accurate mirror of adrenal function. Confirmation of this suggestion has been presented in the present report from two sources. First, rapid detection of changes in plasma 17-OH-CS levels by measurement of parotid fluid values has been demonstrated by the finding of maximal elevation in the parotid fluid concentrations within the first twenty minutes after an intravenous injection of cortisol. Second, individuals who showed adrenal hyper- or hyporesponsiveness to ACTH, as far as plasma 17-OH-CS levels were concerned, demonstrated similar findings in the parotid fluid.

Although in pregnancy, another state associated with elevation of plasma 17-OH-CS, parotid fluid 17-OH-CS were significantly ($P < .02$) elevated, the increase in the mean value was very small (3.0 to 4.2 μg./100 ml.). For comparison, it can be seen in figure 8 that normal men, with mean baseline plasma and parotid fluid 17-OH-CS levels approximating those of the *nonpregnant* women, when given ACTH, had a mean plasma value similar to that of *pregnant women* but a markedly higher mean parotid fluid concentration than shown by the latter group. If it could be shown that only nonprotein-bound 17-OH-CS reach the parotid fluid, then this difference could be ascribed to the low level of nonprotein-bound

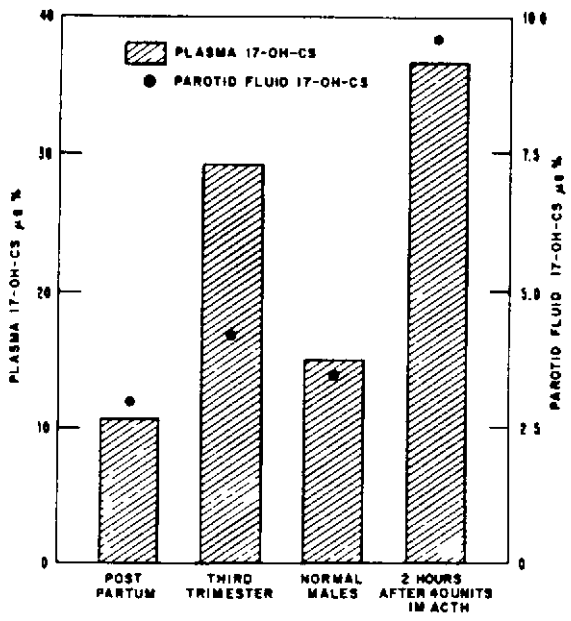

FIGURE 8

Rise in plasma and parotid fluid 17-OH-CS in pregnant patient given ACTH.

cortisol in pregnancy plasma and the high nonprotein-bound plasma cortisol after ACTH treatment, reported by Doe et al. (15). The small but significant increase in nonprotein-bound plasma cortisol found by these workers in pregnancy could then explain the small but significant increase in parotid fluid 17-OH-CS found in pregnancy. Further studies using dialysis technics on concurrently collected plasma and parotid fluid should shed light on this problem.

The finding of similar amounts of cortisol and cortisone in parotid fluid, despite the absence of significant quantities of the latter steroid in plasma, is reminiscent of the findings in amniotic fluid (16, 17). The incubation studies have demonstrated the presence of 11β-hydroxysteroid dehydrogenase activity (18) in dog parotid and skeletal muscle slices. This enzymatic activity is widespread in mammalian tissues (19, 20) and represents the first step in the major pathway of cortisol catabolism.

Thus, it would appear that parotid fluid corticosteroid levels are representative of plasma cortisol concentration, and, if the above speculations are borne out, parotid fluid levels may be a better measure of the physiologically active, nonprotein-bound hormone than the plasma determination.

The results of the present study would be compatible with the hypothesis that filtration analogous to glomerular filtration is the parotid process responsible for the appearance of the corticosteroids, including aldosterone, in the parotid fluid, if it were not for the inability to find corticosteroid conjugates in the fluid. This question requires further study.

Aside from these considerations, this technic is a valuable one because parotid fluid can be obtained in an almost unlimited number of serial samples for endocrine studies when blood sampling has to be limited, as in pediatric subjects.

ADDENDUM

After the completion of this study and during the preparation of the manuscript, an article appeared by Greaves and West (21) reporting that cortisol and cortisone are the major free 17-OH-CS in whole saliva from human females and that they increase in concentration in pregnancy and are completely ultrafiltrable.

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