THE ROLE OF PHOSPHOPYRIDINENUCLEOTIDES IN THE METABOLISM OF CORTISOL BY PERIPHERAL TISSUE

MAX L. SWEAT AND MELVIN J. BRYSON

Department of Obstetrics and Gynecology, University of Utah College of Medicine, Salt Lake City, Utah (U.S.A.)

(Received March 7th, 1960)

SUMMARY

1. Cortisol incubated with muscle tissue is metabolized to cortisone, dihydrocortisol, corticosterone, 11-β-hydroxyandrostenedione, Reichstein's substances E and U, dihydrocortisone, Kendall's Compound A and adrenosterone.

2. The reaction, cortisol → cortisone is TPN specific. DPN appears to inhibit the reaction. No evidence was obtained for the presence of a transhydrogenase in muscle tissue. Reduction at C-4, 5, and C-20 appears to be TPNH specific.

3. Studies of the metabolism of cortisone in muscle and liver indicate that the major product in liver is cortisol. The major product in muscle is Reichstein's substance U.

INTRODUCTION

Since the original observations that DPN plays a role in the metabolism of testosterone by the liver[1–3], the role of phosphopyridine-nucleotides has been studied extensively in the synthesis[4–7], hepatic degradation[5,6,8] and placental metabolism[8–12] of the steroid hormones. The advent of radioactive isotope techniques now makes possible studies of the relationship of these co-factors to the metabolism of steroid hormones in peripheral tissue, which is probably a site of action for several of the steroid hormones. In this study, it has been found that cortisol is metabolized to five products in muscle. One of the products (cortisone) is converted to four additional compounds. Several steroid conversions in muscle and connective tissue appear to be mediated through TPN and TPNH.

EXPERIMENTAL

1.0 g bovine* (psoas major) or mouse leg muscle (approx. 5% connective tissue, estimated histologically) or 0.2 g mouse facia (subdermal loose connective tissue) was incubated in a medium containing sodium phosphate buffer, 0.05 *M*, pH 7.35;

Abbreviations: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide.

* Obtained from slaughter house, kept on ice and teased free of fat and facia. Activity bore a relationship to the time taken for preparation. If too long an interim ensued the preparations were inactive. Age of the animal also seems to be a factor in the degree of activity.

$MgCl_2$, 0.004 M; KCl, 0.025 M; 16,000 counts/min cortisol (specific activity 4.04 μC/mg) or cortisone (specific activity 0.18 μC/mg and 13.9 μC/mg) at 37° for 2 h. Cofactors were employed in concentrations of $1 \cdot 10^{-3}$ and $1 \cdot 10^{-4}$ M. The soluble enzyme of muscle was prepared from the supernatant fraction (32,000 $\times$ g, for 1 h) of a homogenate (1 part tissue, 5 parts above buffer) by fractionating between 10 and 60% saturated ammonium sulfate. A quantity of extract equivalent to 7.5 g of muscle tissue was employed for each experiment.

After incubation, three volumes of acetone were added to the incubation flasks (flasks contained 5–10 ml of incubation mixture) to precipitate the protein. The acetone was subsequently removed by vacuum distillation. The water residue was extracted with chloroform which was evaporated under reduced pressure. The chloroform residue was dissolved in chloroform–methanol (1:1) solution and chromatographed according to the methods of ZAFFARONI[13]. Radioactive areas of the chromatographs were located and analyzed by means of a strip feed counter[14,15]. Specific identification procedures are referred to below. In the oxidation procedures[15], 0.5 mg of chromium trioxide was employed for cleavage of the side chain; 0.1 mg for oxidation of the hydroxyl groups. Dryness of the chromium trioxide was assured by preliminary washing of the crystals with the glacial acetic acid. Control experiments with buffer indicated no steroid conversions.

RESULTS

Presumptive identifications of the radioactive products obtained in the experiments below were based on the following evidences:

Cortisol: Migrates with index cortisol in the chloroform system. Acetylation of combined index and radioactive areas gives compounds which migrate identically with cortisol acetate-21 (chloroform system). Mild oxidation of the acetate results in a compound identical with cortisone acetate-21. Oxidation of the unacetylated compound gives rise to adrenosterone.

Cortisone: Chromatographs with index cortisone in the chloroform system. Elution and acetylation of the combined forms of the steroids (radioactive and non-radioactive) results in derivatives which migrate in the chloroform and benzene systems at an identical rate to that of cortisone acetate. Oxidation of the unacetylated compound gives adrenosterone as a product.

Reichstein's substance E (4-pregnene-11-beta, 17-alpha, 20-beta, 21-tetrol-3-one): Chromatographs near the origin of the chloroform system. When the elution solvent (chloroform) is allowed to drop from the chromatogram (6 h) the radioactivity migrates with Reichstein's substance E. Acetylation of the compound forms a diacetate which migrates at the same rate as substance E-diacetate. Mild oxidation converts the radioactive compound to Kendall's compound E. Strong oxidation converts it to adrenosterone.

20-epi Reichstein's substance E: This compound is slightly more polar than substance E. It forms a diacetate which cannot be distinguished from substance E diacetate when chromatographed in the benzene system (6 h). Mild oxidation of the acetylated compound converts it to substance U acetate. This compound was observed primarily in liver after the incubation of cortisone. BERLINER *et al.*[16] have reported it to be a product of cortisol metabolism in connective tissue.

Reichstein's substance U (4-pregnene-17-alpha, 20-beta, 21-triol-3,11-dione): Migrates between Kendall's compounds E and F in the chloroform (front system.) When the eluent is dropped from the front of the chromatogram (4 h), the compound runs identically with the index compound. Upon acetylation, the compound forms a diacetate which positions itself with substance U-diacetate in the chloroform and benzene systems. Mild oxidation converts the free compound to compound E. Strong oxidation converts the free compound to adrenosterone.

Dihydro F (pregnane-11-beta, 17-alpha, 21-triol-3, 20-dione): This compound chromatographs slightly ahead of compound F in chloroform. When the solvent is dropped, the radioactive compound is resolved from the compound F and migrates identically with the index carrier compound. Acetylation of the radioactive compound forms a monoacetate which migrates identically with the acetate of index dihydrocortisol. Upon mild oxidation of the acetate a slightly less polar compound is formed which corresponds to dihydro compound E acetate.

Dihydro E (pregnane-17-alpha, 21-diol-3, 11, 20-trione): This compound chromatographs in a region slightly ahead of cortisone in the chloroform system. When the solvent is allowed to drop from the front of the chromatogram (3 h), the compound separates from cortisone. Acetylation gives rise to a monoacetate which migrates slightly ahead of cortisone acetate. An index compound was not available for this substance, but the chromatographic characteristics in respect to Kendall's compound E afford strong evidence that it is pregnane-17 alpha, 21-diol-3, 11, 20-trione.

Kendall's compound B (4-pregnene-11-beta, 21-diol-3, 20-dione): In the chloroform and benzene systems, this compound migrates identically with the standard compound. It forms a monoacetate which migrates with compound B acetate. Mild oxidation converts the compound (or its acetate) to Kendall's Compound A (or its respective acetate). Cleavage of the side chain by strong oxidation gives a highly polar compound corresponding to cholanic acid.

Kendall's compound A (4-pregnene-21-ol-3, 11, 20-trione): Chromatography in the benzene and chloroform systems demonstrated this compound to migrate identically with an authentic sample of Kendall's Compound A. A monoacetate is formed upon acetylation and runs at the same rate as the index acetate. Strong oxidation results in a highly polar compound corresponding to cholanic acid.

11-beta-hydroxyandrostene-3, 17-dione: This compound migrates with the non-radioactive carrier compound in the benzene and hexane-benzene systems. Mild oxidation converts it to adrenosterone. It does not form an acetate.

Adrenosterone (4-androstene-3, 11, 17-trione): This compound migrates with the carrier compound in the benzene and hexane–benzene systems. It does not form an acetate. Under the conditions of oxidation described above, this compound is not oxidized to another product.

Fig. 1 is a chromatograph of the steroid products of radioactive cortisol incubated with muscle. A similar spectrum of products has been observed with cultured human[17] and mouse fibroblasts[16]. In the presence of TPN, the respective peaks are elevated, while in the presence of DPN there appears to be an inhibition. A similar effect of TPN has been shown when cortisol is incubated with connective tissue (Fig. 2), albeit the oxidized cofactor does not seem to lend to an increase in the quantity of the high polar compounds in this latter tissue. When a TPNH generating system was incorporated with the connective tissue preparations, the high polar components

were increased in quantity. The magnitude of cortisol metabolism in connective tissue is 3 to 4 times that of the muscle tissue.

To determine whether the specificity of the oxidation of cortisol to cortisone in respect to TPN and DPN was real or due to a membrane barrier, an active soluble extract was prepared and studied. In Fig 3, tracing No. 6 was obtained when no cofactors were added at a pH of 7.4. When TPN was added at this pH, a significant

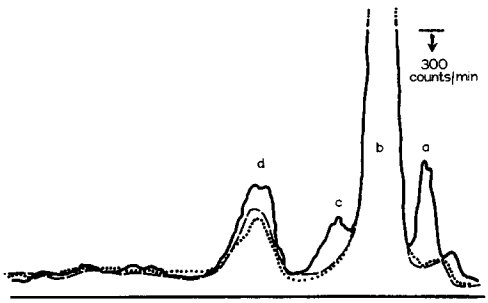

Fig. 1.

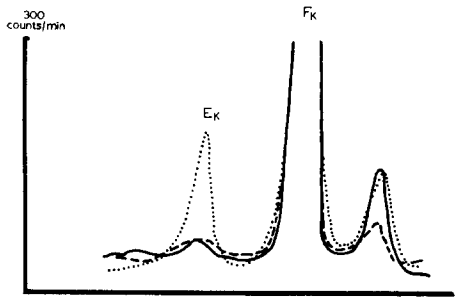

Fig. 2.

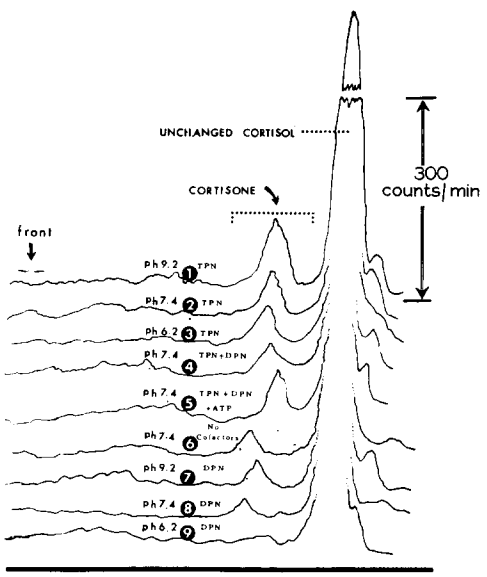

Fig. 3.

Fig. 1. Influence of TPN and DPN on cortisol metabolism by muscle tissue. The tracings are from radiochromatographic recordings obtained from cortisol products initially chromatographed in ZAFFARONI's chloroform–formamide system. The respective peaks were further chromatographed to give evidence of the following compounds: Peak a is largely composed of Reichstein's substance E, peak b is unchanged cortisol, peak c is largely dihydrocortisol and an as yet unidentified compound, and peak d is cortisone. The elevated area between cortisone and the front of the chromatogram contains Kendall's compound B and 11-beta-hydroxyandrostenedione. ——, TPN; ·····, DPN; – – –, control.

Fig. 2. Influence of TPN and DPN on cortisol metabolism in connective tissue. The respective radioactive areas after further chromatography resolve to essentially the same products as those referred to in Fig. 1. Subscript "K" refers to Kendall's steroid designation. ·····, TPN; – – –, DPN; ——, control.

Fig. 3. Influence of TPN and DPN on cortisol metabolism in a soluble extract of muscle tissue incubated at various pH's.

increase in the quantity of cortisone was formed (No. 2). At a pH of 9.2 in the presence of TPN, a large increase was observed. When the pH was lowered, as depicted in tracing No. 3, the quantity of cortisone formed was decreased. When the DPN was added at pH 7.4 and 9.2 (No. 8, No. 7), a small quantity of cortisone was observed; at pH of 6.2 (No. 9), only a trace was observed to be formed. In all experiments in which DPN was added, no increase of the quantity of cortisone was noted over the control. To determine whether a transhydrogenase possibly functioned in this system, DPN and TPN or TPNH in combination were incubated with the extract and radio-

active cortisol. No evidence was found that DPN played an auxiliary role in these reactions (compare No. 4 with No. 6). Spectrophotometric studies also failed to demonstrate an effect of cortisol in the rate of reduction of either TPN or DPN. A suggestion that ATP might play a role in the reaction is seen in the tracing No. 5 in which ATP was added to the preparation together with TPN and DPN. Activity at the C-20 position is greatly diminished in the soluble preparation.

The data depicted in Fig. 3 is evidence that the oxidation of cortisol to cortisone is TPN specific. TPNH appears to be the specific cofactor in the reduction of cortisol at the 4–5 double bond and the C-20 ketone group. Cortisone seems to be the major steroid product of cortisol metabolism in unfortified muscle mince. The addition of TPN induces a small, but significant increase in the cortisone formed. A greater magnitude of increase is noted in the dihydro and C-20 reduced products when exogenous TPN is added. These observations are difficult to evaluate, but suggest a preferential affinity of TPN with the 11-dehydrocortisolase system in the endogenous preparation. The limited effect of exogenously added cofactor on the quantity of cortisone formed may be due to either a steric hindrance at the C-11 position or a limited quantity of the dehydrogenase.

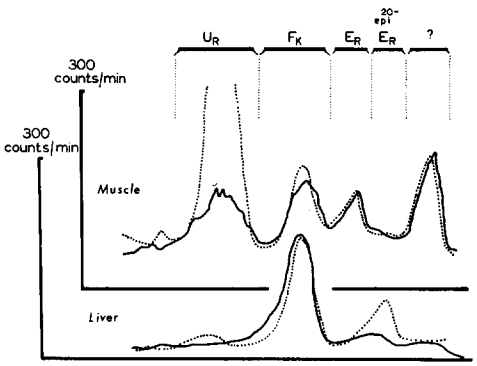

Fig. 4. A comparison of the metabolism of cortisone is muscle and liver. Glucuronic acid conjugation occurs with some steroid products in liver, but has not been detected in muscle tissue. Subscript "R" refers to Reichstein's steroid designation. ———, control; ·····, added TPN.

Studies of the reverse reaction (cortisone → cortisol) in both muscle and liver were also carried out in this investigation. Distinct differences were noted between the two tissues. When [4-^{14}C]cortisone is incubated with mouse muscle tissue, the dominating product is Reichstein's substance U; when incubated with mouse liver tissue the major product is cortisol (Fig. 4). The quantities of cortisol recovered from incubations of cortisone in muscle and liver tissue fortified with either TPN or TPNH do not appear to be significantly different from the controls (Fig. 4). However, the overall production of cortisol in liver seems to be increased with addition of coenzyme as Reichstein's substance E (20-epi), a secondary product of cortisol, is markedly increased. Fortification of the incubation mixtures with coenzyme appreciably increases the quantity of Reichstein's substance U in both tissues. The relative quantities of the products formed in muscle and liver are listed in Table I. Probable interrelations of cortisol, cortisone and their respective products is depicted in Fig. 5.

TABLE I

METABOLISM OF CORTISONE BY MUSCLE AND LIVER TISSUE

	Origin peak		E_R *or Epi* E_R		F_K		U_R	
	%*	*counts/min***	%	*counts/min*	%	*counts/min*	%	*counts/min*
1. Muscle								
Control	20.1	1,382	19.8	1,359	23.3	1,596	36.8	2,524
Addition of TPN								
1-	10.9	1,053	8.2	798	16.4	1,582	64.5	6,239
2-	12.2	937	10.2	774	14.8	1,131	62.8	4,820
2. Liver								
Control	5.6	1,392	13.4	3,308	69.0	17,036	11.9	2,932
Addition of TPN								
1-	6.7	1,949	28.4	8,197	52.0	15,031	12.8	3,702
2-	8.7	2,449	34.1	9,557	44.1	12,368	13.1	3,674

* Relative percentage of lipid soluble products.

** Counts/min were calculated from the area under the curve of the chromatograph as compared to a standard of known radioactivity.

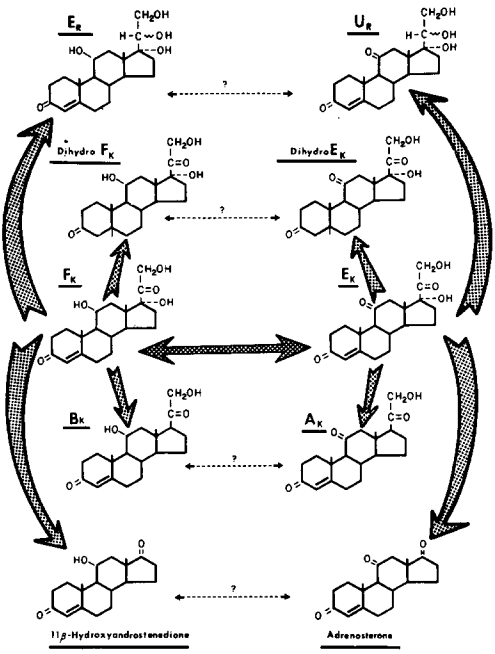

Fig. 5. Interrelations of cortisol and its products in muscle tissue.

DISCUSSION

It is apparent from the above studies that cortisol can be degraded in muscle tissue via at least five separate pathways, namely, cleavage of the C-17 side chain, oxidation at C-11, deoxygenation at C-17 and reduction at C-4, 5, and 20. If the mechanism of action of cortisol is via a chemical process (the possibility exists that cortisol may exert its influence by a physical means without involving a molecular change, *e.g.*, orientation at an interface), it is possible that one or more of these chemical reactions is involved in a key hormonal activation process. As cortisol is known to modify a number of physiological processes (electrolytic, glycogenic and anti-inflammatory

activity, muscle work, etc.), it is also possible that the different chemical conversions are associated with separate physiological processes. That muscle tissue is a target organ of the cortico-steroids is evident from the work of INGLE[18] and SOLOMON AND SAYERS[19]. The relationship between the apparent endogenous affinity of TPN for 11-cortisoldehydrogenase and the mechanism of action of cortisol, as observed in the present experiments, remains to be determined.

BUSH[20] has suggested that the organism tends to maintain the 11-oxygen group of the corticosteroids in the reduced (hydroxyl) form. When 11-keto compounds were administered, a predominance of the 11-hydroxyl products were isolated in the urine. However, FUKUSHIMA *et al.*[21] have recently reported a predominance of the 11-keto urinary derivatives after administration of [4-^{14}C]cortisol. The present studies suggest that reduction of cortisone to cortisol is primarily a hepatic function. In muscle there does not seem to be a marked tendency for C-11 reduction in comparison to the reactions associated with the C-4,5, and 20 positions (Fig. 5). These observations are consistent with the recognition that cortisone administered systematically has comparable effects with cortisol, but when employed locally is much less active[22,23]. In respect to local anti-inflammatory reactions, cortisol has been estimated to be 7 to 8 times as effective as cortisone[24].

ACKNOWLEDGEMENT

This investigation was supported by research grants A-1803 and RG-4720 from the Institute of Arthritis and Metabolic Diseases and the Division of General Medical Sciences, the Public Health Service.

REFERENCES

1 M. L. SWEAT AND L. T. SAMUELS, *J. Biol. Chem.*, 173 (1948) 433.
2 M. L. SWEAT AND L. T. SAMUELS, *J. Biol. Chem.*, 175 (1948) 1.
3 M. L. SWEAT, L. T. SAMUELS AND R. LUMRY, *J. Biol. Chem.*, 185 (1950) 75.
4 M. L. SWEAT AND M. D. LIPSCOMB, *J. Am. Chem. Soc.*, 77 (1955) 5185.
5 G. M. TOMPKINS, *Ann. N. Y. Acad. Sci.*, 82 (1959) 836.
6 P. TALALAY, *Physiol. Revs.*, 37 (1957) 362.
7 G. K. GRANT, *Ann. Repts. on Progr. Chem. (Chem. Soc. London)*, 52 (1955) 316.
8 C. D. KOCHAKIAN, *IV Internat. Congr. Biochem.*, Symposia IV, Pergamon, N.Y., 1959, p. 196.
9 E. D. HAGERMAN AND C. A. VILLEE, *J. Biol. Chem.*, 234 (1959) 2031.
10 P. TALALAY AND H. G. WILLIAMS-ASHMAN, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 15.
11 V. P. HOLLANDER, N. HOLLANDER AND J. D. BROWN, *J. Biol. Chem.*, 234 (1959) 1678.
12 L. J. LANGER AND L. L. ENGEL, *J. Biol. Chem.*, 233 (1958) 583.
13 A. ZAFFARONI, *Recent Progr. in Hormone Research*, 8 (1958) 51.
14 D. L. BERLINER, O. V. DOMINGUEZ AND G. WESTENSKOW, *Anal. Chem.*, 29 (1957) 1797.
15 D. L. BERLINER AND H. A. SALHANICK, *Anal. Chem.*, 28 (1956) 1608.
16 D. L. BERLINER AND T. F. DOUGHERTY, *Soc. Exptl. Biol. Med.*, 98 (1958) 3.
17 M. L. SWEAT, B. I. GROSSER, D. L. BERLINER, H. E. SWIM, C. J. NABORS, JR. AND T. F. DOUGHERTY, *Biochim. Biophys. Acta*, 28 (1958) 595.
18 D. V. INGLE, *J. Clin. Endocrinol.*, 10 (1950) 1312.
19 N. SOLOMON AND G. SAYERS, *Federation Proc.*, 18 (1959) 446.
20 I. E. BUSH AND V. B. MAHESH, *Biochem. J.*, 69 (1958) P. 9.
21 D. K. FUKUSHIMA, H. L. BRADLOW, L. HELLMAN, B. ZUMOFF AND T. F. GALLAGHER, *J. Biol. Chem.*, 235 (1960) 2246.
22 M. ZIFF, E. SCULL, D. FORD, C. MCEWEN AND J. J. BUNIUM, *A. M. A. Arch. Internal. Med.*, 90 (1952) 774.
23 J. L. HOLLANDER, E. M. BROWN, JR., R. A. JESSAR AND C. Y. BROWN, *J. Am. Med. Assoc.*, 147 (1951) 1629.
24 T. F. DOUGHERTY, in *Adrenal Cortex., Trans. of 2nd Conf.*, Josiah Macy, Jr. Fn., N.Y., 1950, p. 88.