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INFLUENCE OF ANABOLIC HORMONES ON PROTEIN METABOLISM IN THE ISOLATED PERFUSED RAT HIND LIMB: A TECHNIQUE FOR CYCLIC CROSS-PERFUSION OF ISOLATED LIMB AND LIVER

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SUMMARY

In the cyclic perfused skinned hind limb, insulin stimulated the incorporation of labeled leucine into protein and the uptake and/or exchange of glycine, alanine, valine, methionine, isoleucine and leucine into skeletal muscle. The combination of insulin and testosterone was more effective in stimulating protein synthesis than insulin. The substituted derivative of testosterone, 17-β-hydroxy-17-α-methyl-2-oxandrostan-3-one (SC11585), was as effective as insulin in stimulating protein synthesis and the uptake and/or exchange of several amino acids. Testosterone was ineffective in increasing protein synthesizing capacity.

A technique for the cyclic cross-perfusion of isolated skinned rat hind limb and liver is described. Under conditions of these cyclic cross-perfusions there is no support for the proposal of a concept of alteration of the testosterone molecule by the liver into an active metabolite which is functional in protein synthesis in skeletal muscle.

INTRODUCTION

Testosterone causes a significant increase in muscular development in the growing male. Both growth and nitrogen retention are accentuated by androgens [1–3]. However, the anabolic properties of testosterone have not always been substantiated by studies utilizing orchidectomy, and there is conflicting evidence as to the effects of androgen on body weight changes in rats [4, 5]. Increased body weight, muscle hypertrophy and accumulation of total body potassium were observed in men treated with methandienone [6]. These changes have been attributed to either a direct anabolic action or the accumulation of intracellular fluid. Thus, the assessment of nitrogen balance is difficult due to many inescapable errors and inevitable problems inherent in available techniques [7]. There are some significant problems in the use of the intact animal in the study of specific hormone actions because the influence of other circulating hormones or substrates cannot be controlled sufficiently. Sirek and Best [8] reported the necessity of insulin for androgens to influence protein biosynthesis; however, Kochakian and Costa [9] refute this claim. The significance of the reports [10–12] indicating the lack of a direct anabolic effect of testosterone in cell free systems on protein biosynthesis in skeletal muscle leads one to the following conclusions: (a) An intact cell membrane may be necessary for testosterone to react with a cytoplasmic receptor to exert its anabolic effect in skeletal muscle [13]. Several studies suggest that steroid hormones may also act on the post-transcriptional regulation of gene expression [14–16]. (b) Testosterone *per se* may not be the active principal in skeletal muscle protein biosynthesis [17]. The effective agent is some transitional degradative metabolite. This would probably involve a significant mediating role of the liver and/or kidney for the metabolism of testosterone. (c) An *in vitro* cell-free system prepared from skeletal muscle is unable to actively incorporate significant amino acids into muscle proteins, due to their long half-life or slow turnover time [18]. (d) Other hormones may be necessary for testosterone to express its protein biosynthetic potential in skeletal muscle. (e) Anabolic hormones may act by counteracting the effects of catabolic steroids at the level of cytoplasmic receptors [17].

This report describes the application of the technique of cyclic cross-perfusion between organs or physiological units and the results of experiments designed to elucidate the mechanism of action of testosterone and other androgens on protein biosynthesis in skeletal muscle of the rat hind limb.

EXPERIMENTAL PROCEDURE

Animals

Hind limb, liver and blood donors were orchidectomized male Sprague–Dawley rats (400–500 g). They were fed Purina Lab Chow and water *ad libitum*. At least 1 week post-orchidectomy recovery was allowed.

Technique of hind limb and liver cross-perfusion

The technique of cross-perfusion between organs or physiological units has not been previously de-

scribed. Livers and hind limbs were from different donors. The methodology of liver removal was according to Veneziale *et al.* [19].

Rats were anesthetized with ether (Anesthesia, Mallinckrodt Inc., St. Louis, MO, U.S.A.) and injected (i.p.) with either sodium amytal (4.7 mg/100 g body weight) or sodium pentobarbital (Nembutal, Abbott Laboratories, Chicago, IL, U.S.A.) (50 mg/ml, 0.35–0.4 ml/rat), for removal of the hind limb. The abdominal cavity was immediately opened from pubis to rib cage and partially eviscerated. 1 ml of heparin (Lipo-heparin, 5000 U/ml, Riker Laboratories, Inc., Northridge, CA, U.S.A.) was infused into the *vena cava* just above the renal vein. The *vena cava* and iliac artery were separated just below the renal vein and artery. The iliolumbar, internal spermatic, inferior epigastric, hypogastric trunk, superior vesical were ligated prior to the removal of the prostate, seminal vesicles, coagulating gland, bladder, and penis. The inferior mesenteric was ligated and the lower intestine and rectum were freed from the surrounding mesentery. Blunt dissection was used to remove the skin from the lower portion of the body to the junction of the femur and the tibia and fibula of the hind limb. The tail was crushed, ligated and severed. A cannula (Intramedic polyethylene tubing, PE 90, Clay Adams, Parsippany, NJ, U.S.A.) was inserted just below the renal arteries nearly to the bifurcation so as to bypass all lumbar arteries. Immediately a glass cannula was inserted into the *vena cava* just below the renal veins and flow was established by applying pressure to the syringe attached to the arterial cannula via a plastic tubing adaptor (size B, Clay Adams). The hind limb was severed just below the kidneys and above the cannulas and was attached to a glass support plate by a wire inserted through the lumbar muscles. The hind limb preparation was inserted into the chamber and attached to the outlet of the reservoir located 100 cm above.

The hind limb and liver were cyclic perfused (perfusion medium recirculated) with Krebs–Ringer bicarbonate (KRB) buffer, containing 3 g bovine serum albumin per 100 ml (Cohn Fraction V, Sigma, St. Louis, MO, U.S.A.) and sufficient rat erythrocytes to give a hematocrit of 10–12%. 20–24 h prior to perfusion, blood was withdrawn from fed orchidectomized rats via heart puncture with a heparinized syringe. The erythrocytes were washed twice with saline before suspending in KRB plus albumin.

The perfusion apparatus was enclosed in a box where humidified air was circulated and maintained at 37°C (Fig. 1). Two modified pumps (Filamatic Vial Filler, National Instrument Co., Baltimore, MD, U.S.A.), rubber diaphragms and one-way Teflon check valves (P575-0332[CV-A], Mallinckrodt, Inc.) were used to move perfusion medium from the pools to alternate reservoirs. Flow rates were in slight excess of 12 ml/min to maintain reservoir levels and allow a minimum bypass to the pools. The perfusion medium was equilibrated with humidified (0.9% saline) O_2–CO_2 (95:5) at two sites, i.e. the oxygenation column over the liver pool and the surface of the glass over the hind limb pool. The gas mixture was withdrawn at four points, i.e. both pools and both reservoirs. Portions of perfusion medium could be removed to sample direct effluent from the liver and hind limb and influent or pool substrate levels for either organ or physiological unit.

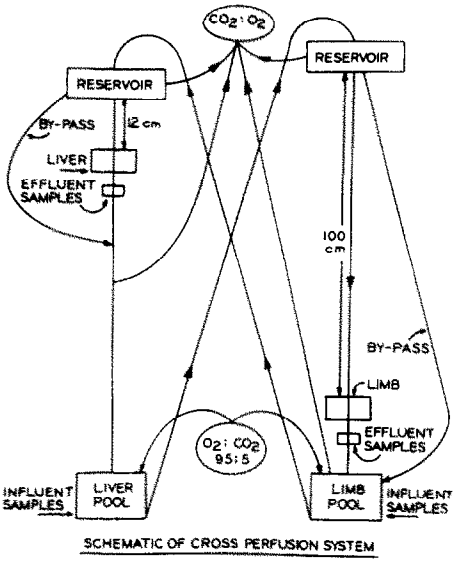

Fig. 1. Schematic of cyclic cross-perfusion system between isolated liver and skinned hind limb.

The pressure at the portal vein and hind limb was 12 and 100 cm of water, respectively. Flow rates of perfusion medium were maintained at 12 ml/min in both hind limb and liver preparations. The total volume of perfusion medium in the recirculation system was 200 ml. The liver was allowed to equilibrate in the system for 60 min and the hind limb for 15 min, prior to the addition of substrate. Single perfusions of hind limb were carried out under identical conditions as cross-perfusions.

Substrates

Glucose was added to the perfusion medium to obtain a concentration of 4.16 mM. The normal fed rat plasma amino acid concentrations (micromolar) were: taurine, 200; aspartate, 35; arginine, 200; proline, 180; glutamate, 200; glycine, 400; alanine, 450; α-aminobutyrate, 15; valine, 200; isoleucine, 100; leucine, 170; phenylalanine, 80; lysine, 400; histidine, 80; tryptophan, 70; glutamine, 350; cysteine, 70; methionine, 70; tyrosine, 70; ornithine, 90; and citrilline, 80, according to Mallette *et al.* [20].

Substrate was prepared by adding glucose and the radioisotopic amino acid to a complete mixture of amino acids suspended in saline (5 ml) at 40 times the desired final concentration. The substrate was added to the perfusion medium in the hind limb pool after equilibration of organs or physiological units (zero time).

Chemicals

Glucose (Dextrose Anhydrous ACS), L-amino acids (MA grade), and bovine serum albumin (Cohn Fraction V) were obtained from Allied Chemical, Palatine, IL, U.S.A., Mann Research Laboratories, New York, NY U.S.A., and Sigma, St. Louis, MO, U.S.A., respectively. L-[U^{14}C]Leucine (240–311 mCi/mmole) was obtained from either Tracer Lab, Waltham, MA, U.S.A., International Chemical and Nuclear Corporation, Irvine, CA, U.S.A., Amersham, Arlington Heights, IL, U.S.A., or Schwarz Mann, Orangeburg, NY, U.S.A. All other chemicals were of the highest chemical purity available.

Hormones

Cyclic cross-perfusions. Insulin (Iletin 40 U/cm^3, Eli Lilly and Co., Indianapolis, IN, U.S.A.) (10 mU/ml of perfusate) and testosterone (17-β-hydroxyandrost-4-en-3-one, Mann Research Laboratories, New York, NY, U.S.A.) (10^{-5} M final concentration) were added at zero time and every 15 min thereafter (six additions) unless otherwise noted. The testosterone was dissolved in *N'-N*-dimethylformide (DMF): 95% ethanol (ETOH) (1:4 ratio) (1.3 mM DMF and 6.55 mM ETOH final concentration at each addition).

Cyclic hind limb perfusions. Biological agents were added at the beginning of the perfusion (zero time): insulin, 10 mU/ml; absolute ethanol (ETOH) as a solvent, 0.43 μM; testosterone, 10^{-5} M; and an oxasteroid, 17-β-hydroxy-17-α-methyl-2-oxandrostan-3-one (SC 11585; MW 276.379) 10^{-5} M (obtained as a gift from Dr R. Pappo, G. D. Searle and Co., Chicago, IL, U.S.A.).

Analysis of perfusion medium. Pool blood aliquots were taken just prior to the addition of substrates and hormones (zero time) and every 15 min thereafter until 90 min had elapsed. In later experiments, both pool and effluent directly from organs or physiological units were taken for glucose determinations (Glucostat, Worthington Biochemical Corp. Freehold, NJ, U.S.A.). At the end of the perfusion, pool blood (20 ml) was centrifuged to remove the erythrocytes and obtain the plasma. Sulfosalicylic acid (7%, w/v) was added to the plasma in a 1:1 ratio and centrifuged to obtain free amino acids. Acidic and basic amino acid concentrations were determined using a Beckman Model 120B Amino Acid Analyzer.

Analysis of liver and muscle

Immediately after cessation of perfusion, a portion of the gastrocnemius and soleus muscle (2.5–3 g) from each hind limb and a portion of the liver (2 g) were quickly frozen and a subsequent protein precipitate was prepared by homogenizing in a Potter–Elvehjem homogenizer with a Teflon pestle in 20 ml cold 5% (w/v) trichloroacetic acid (TCA), followed by washing of the precipitate in 20 ml hot (60°C) 5% (w/v) TCA, 20 ml cold 10% (w/v) TCA, 20 ml hot (60°C) absolute ethanol, 20 ml cold ethanol:ether (3:1) and 20 ml cold ether.

20 μl of the supernatant of each wash was absorbed on membrane filters (100 × 2.5 cm; Radiochemical Centre, Amersham, England, No. 2339) and dried under a heat lamp. Filters were counted in 10 ml of toluene (0.1 g POPOP, 4 g POP/l). No further extractions were required since all free radioactivity was removed. The final precipitate was dried under N_2. Approximately 25 mg ($\pm$2 mg) of dried protein was placed in a glass scintillation vial and 1 ml of a digestor (Soluene, Packard Instrument Co., Downers Grove, IL, U.S.A., or NCS, Amersham, Arlington Heights, IL, U.S.A.) was added and allowed to stand until completely digested. Bray's scintillation solution (18 ml) was added to each vial and 24 h equilibration was allowed in order to reduce random fluorescence. Incorporation of [^{14}C] into protein was expressed as background and quench corrected c.p.m./mg dried total proteins.

Performic oxidation [21], lyophilization and 6 N HCl hydrolysis for 18 h were carried out with a 50 mg pooled sample of extracted dried hind limb protein, in order to determine whether the ^{14}C of leucine was incorporated into protein as leucine. The hydrolyzate was vacuum dried over NaOH and suspended in minimal water. The water soluble amino acids and standards were chromatographed on Whatman 3MM paper with butanol–acetic acid–water (60:15:25) (one dimension, two solvent separations). Leucine and isoleucine were not separated under these conditions. 1 cm paper strips were counted in 10 ml toluene. Radioactivity of N- and C-terminal amino acids were determined according to Suttie [22]. The ^{14}C in protein reflects the total amount of protein synthesis occurring during the perfusion because of the slow turnover of amino acids in muscle protein. Where appropriate, data were analyzed statistically by analysis of variance, and multiple range test.

RESULTS

Cyclic cross-perfusions

Changes in glucose concentration. The basic perfusion medium was KRB and erythrocytes (hematocrit, 10–12%). Erythrocytes in the presence of glucose and amino acids, but without the liver and limb present, removed glucose (Δ – 1.23 μmol/min) from the perfusion medium (not shown). In the presence of the liver and hind limb but in the absence of glucose and amino acids, the release of glucose was observed in both pools (Table 1). In the presence of an initial glucose concentration of 4.16 mM and a mixture of amino acids there was a significant net uptake of glucose from both pools. The addition of DMF:ETOH to the perfusate in the presence of substrates inhibited the disappearance of glucose from the perfusate or increased the production of glucose via gluconeogenesis or glyogenolysis. Insulin stimulated the uptake of glucose from both pools whereas testosterone caused a release of glucose into the perfusate. Insulin in combination with testosterone effec-

Table 1. Changes in pool or influent glucose levels and radioactivity in proteins from the isolated liver and the skinned hind limb of fed orchidectomized rats in a cyclic cross-perfusion system with glucose and amino acids as substrate[1]

Treatment[2]	Δ Glucose[3] (μmoles/min) Liver	Limb	c.p.m./mg protein Liver	Limb
None (2)	+2.71 ± 0.5	+3.52 ± 1.3	—	—
Substrate (2)	−3.33 ± 0.2	−3.08 ± 0.2	518 ± 16	61 ± 4
Substrate, solvent (2)	−2.10 ± 0.4	−0.37 ± 0.1	643 ± 7	50 ± 2
Substrate, solvent, insulin (2)	−4.81 ± 1.4	−4.93 ± 1.6	584 ± 2	75 ± 7
Substrate, solvent, testosterone (4)	+6.60 ± 0.8	+7.83 ± 1.5	403 ± 113	46 ± 13
Substrate, solvent, testosterone, insulin (2)	−6.97 ± 1.2	−8.57 ± 0.7	326 ± 33	58 ± 20

[1] Mean ± standard deviation.

[2] Solvent (dimethylformide, 1.3 mM: ethanol, 6.55 mM), insulin (10 mU/ml) and testosterone (10^{-5} M) were added to the perfusion medium six times—zero time and every 15 min thereafter. Perfusion was terminated after 90 min. Substrates (4.16 mM glucose, amino acid and nitrogenous compound mixture, 4 μCi L-[U-^{14}C]leucine) was added at zero time. Numbers in parentheses denote number of cross-perfusions.

[3] Δ glucose, + denotes release into perfusion medium and − denotes uptake from perfusion medium.

tively inhibited the hyperglycemic effect of testosterone. The concentration of ETOH (6.55 mM) at each addition was less than reported values for the acute alcoholic (21.7 mM). In addition, prolonged inhalation of DMF vapors (100 ppm) are known to cause liver injury in rats and may have affected liver or muscle metabolism in these cross-perfusions.

Incorporation of labeled amino acid

Incorporation of [^{14}C]leucine into protein isolated from the liver and hind limb (Table 1) of cross-perfusions indicates that insulin stimulates protein synthesis in the muscle but not in the liver. Testosterone failed to stimulate protein synthesis above control values in the liver and apparently reduced incorporation in the muscle. Insulin and testosterone in combination were not as effective in stimulation of isotope incorporation into muscle and liver as was insulin.

Cyclic hind limb perfusions

Glucose utilization. In order to evaluate the action of anabolic hormones *per se* on skeletal muscle, a minimal amount of absolute ethanol was used to dissolve the steroids. Pool glucose levels were not significantly altered by the addition of ethanol (0.43 μM) to the perfusion medium with substrate (Fig. 2A). Testosterone or SC11585 at the same concentration did not change glucose utilization as compared to the addition of ethanol or the control (Fig. 2B). Insulin significantly increased the rate of utilization of glucose, which was not influenced by the further addition of ethanol containing testosterone or SC11585 (Fig. 2C).

Amino acid incorporation. The amount of radioactivity incorporated into protein of the hind limb is illustrated in Fig. 3. There was no significant increase ($P > 0.05$) in incorporation of L-[U-^{14}C]leucine into protein by the addition of ethanol or testosterone to the substrate. Neither insulin nor SC11585 significantly ($P > 0.05$) increased incorporation of labeled amino acid when added to the substrate. Insulin and SC11585 given simultaneously significantly ($P < 0.05$) increased incorporation above substrate but did not significantly ($P > 0.05$) increase incorporation above either hormone used. The combination of insulin and testosterone increased incorporation significantly ($P < 0.05$) when compared to substrate or testosterone but not when compared to insulin. There was a tendency for insulin to increase the incorporation of L-[U-^{14}C]leucine above the level of the control and to increase still further the incorporation when in combination with testosterone or SC11585.

Performic oxidation, HCl hydrolysis, paper chromatography, and radioactive determinations indicated that leucine was the only radioactive amino acid detected in the hind limb protein. Although leucine and isoleucine were not separated, there is no known metabolic conversion of leucine to isoleucine which would not involve the labeling of other amino acids. Radioactivity was present in only 3% and 11% of the N-terminal and C-terminal amino acids, respectively, proving that ^{14}C from leucine was incorporated into protein as leucine and was not due to exchange of terminal groups.

Analysis of perfusion medium for amino acids and related compounds and the tests for significance are shown in Table 2. Only the following compounds were significantly changed or tended to be affected by treatments.

Glycine. Insulin or insulin plus testosterone increased the uptake of glycine by the hind limb musculature above that of substrate only ($P < 0.05$).

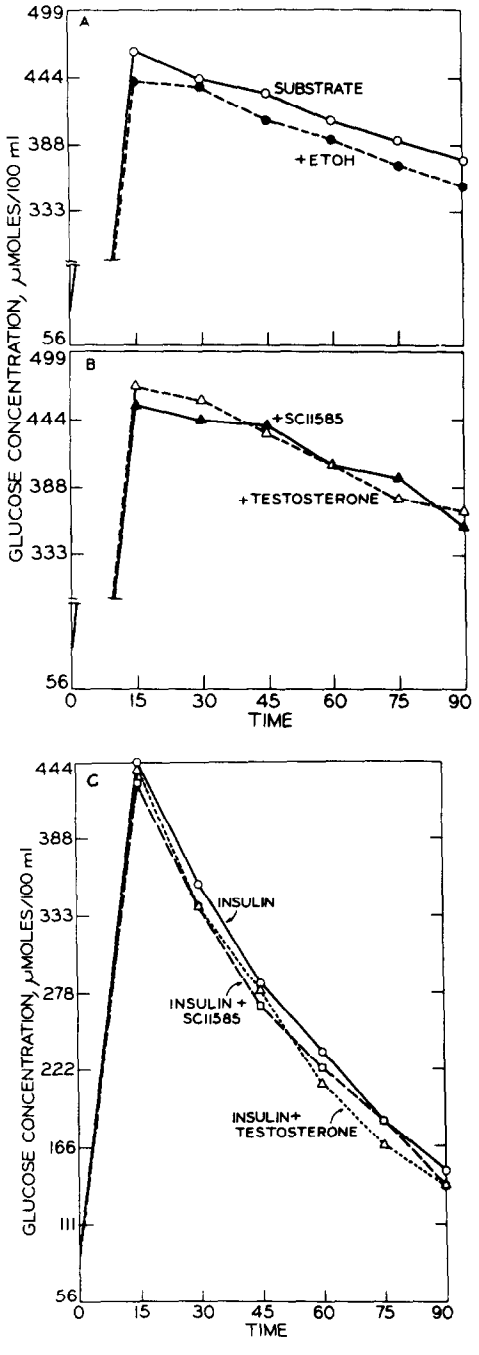

Fig. 2. Changes in pool glucose concentration in the isolated cyclic perfused skinned hind limb of the fed orchidectomized rat. Solvent, hormones, substrates and hind limbs are identical to those in Table 2 and Fig. 3. Standard deviations in glucose concentrations for 0, 15, 30, 45, 60, 75 and 90 min according to treatment with number of hind limbs in parentheses are as follows: Substrate (3): 14.9, 13.6, 2.9, 13.6, 8.6, 16.8 and 15.6 μmol/dl; Substrate, ETOH (4): 32.7, 32.4, 35.5, 34.1, 26.6, 35.4 and 42.1 μmol/dl; Substrate, ETOH, testosterone (4): 23.4, 45.4, 47.3, 51.6, 51.2, 49.2 and 47.8 μmol/dl; Substrate, ETOH, SC11585 (4): 6.8, 17.5, 15.6, 30.3, 37.7, 29.6 and 31.7 μmol/dl; Substrate, ETOH, insulin (6): 23.6, 40.0, 40.8, 31.9, 27.0, 23.6 and 30.6 μmol/dl; Substrate, ETOH, insulin, testosterone (5): 22.4, 29.0, 34.7, 32.5, 21.1, 29.0 and 33.9 μmol/dl; Substrate, ETOH, insulin, SC11585 (5): 19.0, 26.5, 16.1, 15.9, 19.1, 12.3 and 18.7 μmol/dl.

Alanine. All hormones and ethanol additions stimulated ($P < 0.05$) the uptake of alanine by the muscle when compared to substrate. The addition of SC11585 stimulated ($P < 0.05$) uptake above that of the ethanol. Insulin increased uptake to a greater extent than did SC11585; however, the combination of testosterone with insulin had no influence on uptake above insulin. If SC11585 was added with insulin, uptake was greater ($P < 0.05$) than insulin alone; however, when comparing the values, there was no difference in the effectiveness of either SC11585 or testosterone with insulin.

Valine. Insulin and/or SC11585 increased ($P < 0.05$) the uptake of valine by the muscle above that of substrate or ethanol. Uptake was stimulated ($P < 0.05$) to a greater extent by SC11585 than by testosterone or insulin. There was no difference in the effectiveness of insulin alone or when combined with either testosterone or SC11585.

Methionine. All hormones except testosterone increased ($P < 0.05$) the uptake of methionine by the muscle above that of substrate or substrate and ethanol. Insulin and SC11585 were similar as to stimulatory ability; however, combinations of either testosterone or SC11585 and insulin produced the maximum uptake ($P < 0.05$).

Isoleucine. Insulin in combination with testosterone or SC11585 and SC11585 alone increased the uptake of isoleucine by the muscle to a greater extent ($P < 0.05$) than substrate and ethanol but not substrate alone.

Leucine. The uptake of leucine by the muscle from the perfusion medium correlated with the incorporation of ^{14}C from leucine. The order of leucine uptake was as follows: SC11585 and insulin > testosterone and insulin > SC11585 > insulin > testosterone > ethanol > substrate alone. A greater ($P < 0.05$) uptake of leucine occurred when SC11585 was combined with insulin than with insulin alone; however, SC11585 was as effective when used alone as when combined with insulin. Testosterone exerted similar effects when combined with insulin; however, the difference only approached significance ($P > 0.05$). Although insulin increased the uptake above substrate, the difference was not significant ($P > 0.05$).

Glutamine/asparagine. All hormone and ethanol additions tended to decrease the amount of glutamine and/or asparagine in the perfusion medium but the reduction was not significant ($P > 0.05$).

Ammonia. The most anabolic combination of hormones tended to reduce the amount of ammonia in the perfusion medium, but the reductions were not statistically significant ($P > 0.05$).

DISCUSSION

Changes in perfusate glucose

Cyclic cross-perfusions. Ethanol and DMF induced the release and/or inhibited the uptake of glucose in the fed liver and hind limb cross-perfusions in the

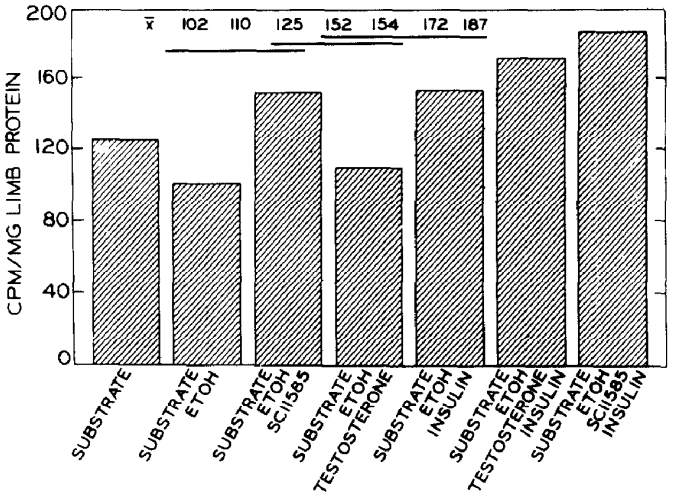

Fig. 3. Incorporation of labeled leucine into trichloroacetic acid-precipitated protein from striated muscle of the isolated cyclic perfused skinned hind limb of the fed orchidectomized rat. Solvent, hormones, substrates and hind limbs are identical to those in Table 2 and Fig. 2. Values underlined by the same line do not differ significantly ($P > 0.05$). Numbers in parentheses in Table 2 or Fig. 2 denote number of hind limbs.

absence of substrates (not shown) or in the presence of glucose and a mixture of amino acids. Testosterone significantly increased glucose release into the perfusate in the absence (not shown) or the presence of substrates. In contrast to the results of cross-perfusions, the addition of testosterone in the absence of substrates failed to stimulate glycogenolysis in the cyclic perfused fed liver or affect the uptake of glucose in the cyclic perfused hind limb (not shown). This glycogenolytic and/or glucogenic activity by testosterone has not been previously reported. Testosterone may increase the oxidation of ethanol through the hepatic microsomal ethanol oxidizing system (MEOS) utilizing NADPH and subsequently glycogenolysis, although this system is considered to be of minor importance. However, Lieber and De Carli [23] have reported the oxidation of ethanol by the MEOS to be significantly greater in male rats than females. The oxidation of testosterone to androstenedione by β-hydroxysteroid dehydrogenase could also generate a more reduced state in the liver, although this may be of little significance because of relatively slow metabolism. This difference between cyclic perfused and cyclic cross-perfused physiological units may reflect some form of tissue communication, but the results can not be clearly explained at this time.

The mixture of amino acids would supply gluconeogenic substrates which would contribute to the total glucose pool, thereby reducing the response to insulin. The formation of glucose from these substrates was also evident in the presence of testosterone. In fact when testosterone was administered with insulin in the presence of glucose and an amino acid mixture, the uptake of glucose was accentuated. Sirek and Best [8] and Bergamini [24] failed to find augmentation by these two hormones on glucose uptake in other tissues.

Cyclic hind limb perfusions. Ethanol (0.43 μM) did not significantly increase the uptake of glucose by the hind limb musculature in the presence of substrates, whereas insulin stimulated the uptake of glucose across the cell membrane, as has been reported by Ruderman *et al.* [25], Jefferson *et al.* [26], Goodman *et al.* [27], Reimer *et al.* [28] and Berger *et al.* [29]. The rate of glucose uptake in the isolated perfused hind limb with substrates and androgens but without insulin was similar to control levels. Bergamini *et al.* [30] reported that castration caused a rapid fall in glycogen content of the rat levator ani muscle and androgen injections restored glycogen via increased sugar transport. The use of the levator ani muscle as an indicator of anabolic or myotrophic activity of androgens has been severely criticized [31]. The relationship between androgenic and anabolic sensitivity of the levator ani muscle as compared to skeletal muscle may account for this difference in response. In contrast to the cross-perfusions, androgens did not augment the insulin response on blood glucose in the isolated cyclic perfused hind limb. Similar results were observed by Sirek and Best [8] and Kochakian and Costa [9] in the castrated dog. The observed augmentation of glucose uptake in the cross-perfusion system by testosterone and insulin could reflect the relationship between physiological units, the effect of different solvents or the higher concentration of hormones.

Incorporation of radioactivity

Insulin stimulated protein synthesis in the muscle of the hind limb but not in the liver from cyclic cross-perfusions. This was also evident in the cyclic perfused hind limb ($P < 0.5$) as reported by Jefferson *et al.* [26]. The addition of either testosterone or SC11585 with insulin was more effective in stimulating protein synthesis in the cyclic perfused hind limb

Table 2. The effect of hormones on amino acid concentration after cyclic perfusion through the isolated skinned hind limb of the fed orchidectomized rats

Treatments[1]	Micromoles per ml					
	Glycine	Alanine	Valine	Methionine	Isoleucine	Leucine
Substrate (3)[2]	$0.315^{bcd} \pm 0.009$	0.421 ± 0.015	$0.123^{c} \pm 0.009$	$0.048^{c} \pm 0.002$	$0.053^{ab} \pm 0.008$	$0.095^{c} \pm 0.001$
Substrate, ETOH (4)	$0.349^{d} \pm 0.036$	$0.413^{c} \pm 0.033$	$0.123^{c} \pm 0.001$	$0.048^{c} \pm 0.004$	$0.058^{b} \pm 0.006$	$0.094^{c} \pm 0.009$
Substrate, ETOH, Testosterone (4)	$0.328^{cd} \pm 0.044$	$0.410^{c} \pm 0.045$	$0.119^{bc} \pm 0.017$	$0.045^{bc} \pm 0.003$	$0.057^{b} \pm 0.009$	$0.093^{c} \pm 0.012$
Substrate, ETOH, SC11585 (4)	$0.292^{abc} \pm 0.017$	$0.338^{d} \pm 0.048$	$0.093^{a} \pm 0.018$	$0.039^{ab} \pm 0.004$	$0.044^{a} \pm 0.008$	$0.073^{ab} \pm 0.009$
Substrate, ETOH, Insulin (6)	$0.278^{a} \pm 0.017$	$0.303^{b} \pm 0.030$	$0.116^{bc} \pm 0.004$	$0.041^{ab} \pm 0.003$	$0.052^{ab} \pm 0.004$	$0.083^{bc} \pm 0.006$
Substrate, ETOH, Insulin, Testosterone (5)	$0.276^{a} \pm 0.038$	$0.295^{ab} \pm 0.021$	$0.101^{ab} \pm 0.014$	$0.038^{a} \pm 0.008$	$0.043^{a} \pm 0.002$	$0.070^{ab} \pm 0.007$
Substrate, ETOH, Insulin, SC11585 (5)	$0.280^{ab} \pm 0.020$	$0.299^{a} \pm 0.017$	$0.108^{abc} \pm 0.007$	$0.038^{a} \pm 0.003$	$0.046^{a} \pm 0.005$	$0.069^{a} \pm 0.010$

[a,b,c,d] Values with the same superscript do not differ significantly ($P > 0.05$). Mean $\pm$ standard deviation.

[1] Solvent (ethanol, 0.43 μM), insulin (10 mU/ml) and testosterone or SC11585 (10^{-5} M) were added to the perfusion medium only once—zero time. Substrates (4.16 mM glucose, amino acid and nitrogenous compound mixture—initial concentration: glycine 0.4, alanine 0.45, valine 0.2, methionine 0.07, isoleucine 0.1, leucine 0.17 μmoles/ml–4 μCi L-[U-^{14}C]leucine) was added at zero time. Perfusion was terminated after 90 min.

[2] Numbers in parentheses denote number of hind limb perfusions.

than insulin. This supports the hypothesis that insulin is necessary for testosterone to be anabolically active as claimed by Sirek and Best [8]. However, this was not the case in the cyclic cross-perfusions. The substituted derivative of testosterone, SC11585, was as effective as insulin in stimulating protein synthesis in the cyclic perfused hind limb. Substitution of specific groups of testosterone have been shown to enhance myotrophic rather than androgenic activity in intact animals and humans [32]. In cyclic perfused hind limb, the effect of insulin was synergistic when combined with testosterone and additive when combined with SC11585. Testosterone was ineffective in stimulating protein synthesis in the muscle of either the cyclic perfused hind limb or the cyclic cross-perfused hind limb, as has been reported in the orchidectomized rat *in vivo* [5]. Under the experimental conditions of cyclic cross-perfusion between liver and hind limb, there is no support for the concept of alteration of the testosterone molecule by the liver into an active metabolite which is myotropically active in skeletal muscle.

Amino acid uptake by the cyclic perfused hind limb

Insulin stimulated the uptake and/or exchange of glycine, alanine, valine, methionine, isoleucine and leucine from the perfusion medium of the cyclic perfused hind limb. Branched chain amino acids (leucine, isoleucine, valine) were responsive to the action of insulin in the muscle of humans [33, 34]. Alanine release was inhibited with insulin in intact humans [33] and in the fed or fasted perfused rat hind limb [35]. In contrast to the uptake of amino acids in the experiments reported here, Felig *et al.* [36] and Ruderman and Berger [35] have reported the release of the "whole spectrum" of plasma amino acids in intact humans and perfused hind limb, respectively. The increased uptake could be due to the inclusion of adequate glucose and amino acids in the perfusion medium. Alanine [37, 38], glycine [39], and methionine [40] uptake in the isolated rat diaphragm muscle was stimulated by insulin. However, the amino acid transport systems described by Kipnis and Parrish [41] for diaphragm under the influence of insulin did not appear similar to those shown here for the cyclic perfused hind limb.

CONCLUSION

The observed differences in uptake of amino acids and the incorporation of leucine into protein in these isolated hind limb perfusions may reflect a system that was more responsive to hormonal stimulation than those reported for diaphragm *in vitro*, or those *in vivo* studies in which there were unknown quantities of circulating hormones. However, isolated hind limbs from young orchidectomized male rats (140–160 g) in the actively growing stage might be stimulated to a greater extent than those of older rats used in these experiments.

Cross-perfusions between or among physiological organs or units may be of importance in resolving the problems of hormone involvement in the regulation of metabolism. Intimate *in vivo* associations of liver and other organs or tissues such as those reported by Horlick and DeLuca [42] for the regulation of calcium uptake may be discovered and studied by the *in vitro* cross-perfusion technique. Under the conditions of these experiments there is no support for the concept of alteration of the testosterone molecule by the liver into an active metabolite which is functional in protein synthesis in skeletal muscle.

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