The effect of steroids and ammonium chloride acidosis on phosphoenolpyruvate carboxykinase in rat kidney cortex: *I. Differentiation of the inductive processes and characterization of enzyme activities*

I. D. Longshaw, C. I. Pogson

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Phosphoenolpyruvate carboxykinase activities in all conditions investigated show similar behavior in dilute extracts: these experiments involved antibody titration, stability studies, and molecular weight determinations on sucrose gradients.

The molecular weight of phosphoenolpyruvate carboxykinase was also studied in undiluted extracts prepared by high-speed centrifugation; values were determined from sedimentation data obtained with a moving-partition cell as described by Yphantis and Waugh. Under these conditions, the apparent molecular weight of phosphoenolpyruvate carboxykinase was increased from 83,000 to 128,000 by ammonium chloride acidosis.

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I. DIFFERENTIATION OF THE INDUCTIVE PROCESSES AND CHARACTERIZATION OF ENZYME ACTIVITIES

I. D. LONGSHAW and C. I. POGSON

From the Department of Biochemistry, University of Bristol, The Medical School, Bristol, United Kingdom

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INTRODUCTION

An important aspect of the response of the kidney to metabolic acidosis is an increased urinary excretion of ammonia (1, 2). This increased production of ammonia is accompanied by a simultaneous increase in glucose synthesis, as measured in experiments with kidney cortex slices from acidotic rats incubated with various C_4 precursors (3). Conversely, both ammonia production and gluconeogenesis from C_4 precursors are reduced after intragastric administration of $NaHCO_3$ (3). Since glutamate is reported to inhibit the activity of glutaminase I (4, 5), Goodman, Fuisz, and Cahill (3) have suggested that the stimulation of gluconeogenesis by acidosis may reduce the total glutamate concentration and thereby stimulate breakdown of glutamine, the major source of excreted ammonia (6). Furthermore, since acidosis leads to a stimulation of gluconeogenesis from C_4 precursors but not from fructose or glycerol, these workers postulated that the site of regulation lies between oxaloacetate and triose phosphate (3). Although it has more recently been demonstrated that ammoniagenesis and gluconeogenesis may be dissociated under certain conditions (7–9), the hypothesis regarding the locus of regulation of gluconeogenesis at a point "distal" to oxaloacetate is supported by measurements of intermediates in freeze-clamped normal and acidotic rat kidneys (10); these observations indicate the presence of a crossover at the level of phosphoenolpyruvate carboxykinase (PE-PCK).[1] In addition, Alleyne and Scullard (11) have

[1] *Abbreviations used in this paper:* LDH, lactate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase; TIM, triosephosphate isomerase.

shown that total assayable renal PEPCK is increased in the acidosis state. This increase in total activity is not blocked by actinomycin D or ethionine, in contrast with that produced by administration of the synthetic glucocorticoid, triamcinolone (9α-fluoro-16α,17α-isopropylidenedioxy-1-dehydrocorticosterone) (12). In this paper we report further evidence that PEPCK induction in acidosis differs qualitatively from that due to administration of steroid, and show that such changes may be associated with increases in enzyme molecular weight; a hypothesis of PEPCK regulation in rat kidney, based on these data, is presented.

METHODS

Animals

Male Wistar rats were used throughout. Weights are given with other experimental details. Rats were fed diet 41B (13), and were allowed water, or 0.18% NaCl, ad lib. 0.18% NaCl replaced water in experiments involving NH₄-Cl-induced acidosis, a procedure which is reported to lead to sodium loss (11); in these experiments, NaCl was given to both controls and·experimental animals.

Tissue extractions

Animals were killed by decapitation. The kidneys were rapidly removed, freed of capsules and connective tissue, blotted, and weighed. One kidney from each animal was then homogenized in 9 vol of extraction medium in a motor-driven all-glass tissue grinder (Kontes Glass Co., Vineland, N. J.) for 1 min at 0°C. Liver extracts were prepared by analogous procedures. The extraction medium consisted of either 0.25 M sucrose (in antibody titration experiments) or 0.154 M KCl, 5 mM-2-mercaptoethanol (most other experiments).

Such homogenates were centrifuged in an Eppendorf 3200 centrifuge (V. A. Howe Ltd., London, U. K.) at 12,000 g for 8 min at 4°C. The supernates were decanted and used immediately as described below.

Undiluted extracts were prepared by homogenization of the tissue at 0°C in the absence of extracting medium, followed by centrifugation at 105,000 g for 2 hr at 4°C in a Beckman L-2 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The top clear layer of the supernate was carefully removed with a Pasteur pipette and was used immediately.

Antibody titrations

The purified antibody employed in these experiments was a kind gift from Dr. R. W. Hanson; it was prepared in rabbits against purified rat liver "cytosolic" PEPCK (14). In preliminary experiments, liver and kidney supernatant fractions were prepared by pooling tissues from three to four animals. In other experiments, extracts were prepared from kidneys of 400-g rats.

Animals were rendered acidotic by a single intragastric administration (12 hr previously) of 2.5 ml of 0.4 M NH₄-Cl/100 g body weight; controls received a similar dose of 0.4 M NaCl. Triamcinolone was also given 12 hr previously by intraperitoneal injection of a suitable suspension in 0.9%

NaCl (0.5 mg/100 g body weight); controls were injected with 0.9% NaCl.

Final extracts were prepared by mixture of equal volumes of supernates derived from the kidneys of the three animals in each group.

Antibody titration mixtures contained 100 μl of extract and 75 μl 0.9% NaCl, together with various amounts of antibody. The total serum content was adjusted to 75 μl by appropriate admixture of normal rabbit serum. After incubation at 37°C for 30 min, samples were left at 4°C for 3 hr to allow complete precipitation of the antigen-antibody complex. The precipitates were removed by centrifugation at 3,000 g for 5 min and PEPCK activity in the supernates was assayed as described elsewhere. Results are expressed as total PEPCK remaining in the supernatant fluid after precipitation.

Molecular weight determinations

The molecular weight of PEPCK in kidney extracts from normal and 48-hr acidotic 400-g rats (four administrations of 2.5 ml 0.4 M NH₄Cl/100 g body weight at 12-hr intervals) was investigated by two techniques.

Martin and Ames method (15). Extracts were prepared in 9 vol of 50 mM Tris Cl, pH 7.5. 100 μl portions of the supernates after centrifuging were layered on 4.5 ml of 5–20% continuous sucrose gradients. Gradients were centrifuged at 100,000 g for 15 hr at 1°C in the L-2 preparative ultracentrifuge. After spinning, each gradient was separated into 5-drop fractions (80 μl approximately). PEPCK and lactate dehydrogenase (LDH) activities were measured in suitable portions from each fraction.

Separation cell method. Undiluted extracts from kidneys of normal and 48-hr acidotic 400-g rats were centrifuged in a partition cell as described by Yphantis and Waugh (16) at 59,780 rpm for 45 min at 20°C in a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The procedure followed was as given by Schachman (17). Results were calculated from an empirical standard curve derived from the activities of the three "marker" enzymes (catalase, LDH, and triosephosphate isomerase [TIM]) both in the "top" compartment after centrifugation and in the original samples.

Induction studies

Eight groups of 130-g animals were used. Injections or administrations were carried out at 0, 12, 24, and 36 hr from the start of the experiment. Animals were killed at 48 hr (0900 hr), and extracts prepared for assay as described above.

Triamcinolone, as a suspension in 0.9% NaCl, was injected at a level of 0.5 mg per animal; acidosis was induced and sustained by intragastric administration of 2.5 ml of 0.4 M NH₄Cl/100 g body weight. Actinomycin D (18 μg, in 0.9% NaCl) was given by intraperitoneal injection 30 min before all other treatments.

Appropriate controls (sham injections with 0.9% NaCl and sham feeding with 0.4 M NaCl) were carried out throughout.

Assay procedures

PEPCK was assayed in the direction of phosphoenol-pyruvate synthesis by the method of Seubert and Huth (18). Lactate dehydrogenase (19) and catalase (20) were

estimated by standard procedures. Triosephosphate isomerase activity was measured by following NADH oxidation in a medium containing 75 mM triethanolamine HCl pH 7.5, 4.5 mM MgSO$_4$, 1 mM-D-glyceraldehyde-3-phosphate and excess L-glycerol-3-phosphate dehydrogenase. One unit of enzyme activity is taken as that which catalyses the conversion of 1 μmole of substrate per min at 37°C (PEPCK) or 30°C (LDH and TIM).

Materials

Triethanolamine HCl, oxaloacetic acid, glyceraldehyde-3-phosphate, NADH, ATP, ADP, AMP, and L-glycerol-3-phosphate dehydrogenase were from the Boehringer Corp. (London) Ltd. Tris, as Trizma base, and 2-mercaptoethanol were from Sigma Chemical Co., St. Louis, Mo. Actinomycin D was from Calbiochem, Los Angeles, Calif. Triamcinolone acetonide (Adcortyl) was a kind gift from E. R. Squibb & Sons Ltd., Speke, Liverpool, U. K.

Free glyceraldehyde-3-phosphate was generated from the diethyl acetal according to Racker, Klybas, and Schramm (21), and inosine triphosphate (ITP) was prepared by diazotization of ATP as described by Kaplan (22).

RESULTS

Induction studies. As previously observed (12) renal PEPCK activity is increased both by steroid administration and in acidosis (Fig. 1). Preliminary experiments indicated that the doses of steroid and NH$_4$Cl given in these studies are sufficient to elicit the maximum response under the conditions prevailing. Despite this observation, activities in animals given both steroid and NH$_4$Cl simultaneously exhibited an additive response to these treatments (Fig. 1). The additive nature of the response was invariably observed however the treatment was administered. This included experiments in which triamcinolone was given for 2 days followed by NH$_4$Cl for a further 2 days before killing.

Actinomycin D is an inhibitor of mRNA transcription (23), and is thus expected to block all increases in enzyme activity which require *de novo* mRNA synthesis. In the experiment shown in Fig. 1, actinomycin D inhibited the steroid-induced increment in PEPCK activity by 82% ($P < 0.001$), but failed to reduce significantly the increment due to acidosis. Such reduction in activity as was observed could be correlated with the similar slight decrease in PEPCK activity in control animals treated with actinomycin D. In animals treated with both steroid and NH$_4$Cl, actinomycin D effected a 48% reduction ($P < 0.001$) of the increment in total renal PEPCK. This reduction in activity corresponded closely to the increment observed with triamcinolone alone. It also indicates that the efficacy of actinomycin D is in no way impaired in the acidotic animal.

These data suggest that PEPCK may possibly occur in at least two distinct forms in rat kidney cortex. This possibility was investigated in experiments in-
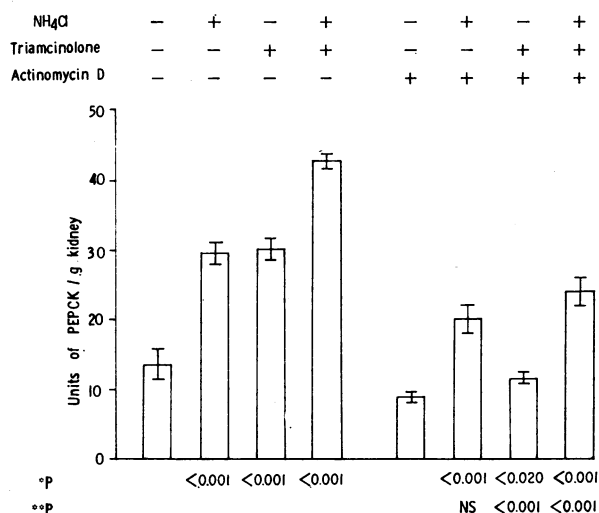

FIGURE 1 Effects of steroid, acidosis, and actinomycin D on renal PEPCK activity. Animals were killed at 48 hr from the start of the experiment. Extraction procedures and dosing protocols were as described in the text. Results are expressed as the means of six observations ±SEM. *P, significance of the differences between the means in treated animals and those in appropriate control groups. **P, significance of the differences between the mean increments in actinomycin D-treated animals and the mean increments in animals similarly treated, but not given actinomycin D.

volving molecular weight determinations, antibody titration, and stability studies.

Molecular weight determinations. Similar sucrose gradient patterns were seen with PEPCK from normal and acidotic kidneys. The molecular weight of PEPCK was calculated to be 74,000 for "acidotic" and 67,000 for "normal" rat kidney PEPCK (Fig. 2). These values
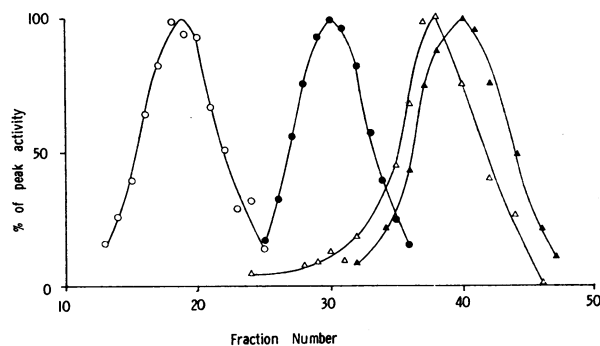

FIGURE 2 Sucrose gradient fractionation of kidney cortex extract from acidotic rats. Procedure was as described in the text. ○, catalase; ●, lactate dehydrogenase; △, PEPCK; ▲, triosephosphate isomerase. The molecular weights of the marker enzymes were taken as, catalase 247,000 (30), lactate dehydrogenase 126,000 (31), and triosephosphate isomerase 50,000 (32, C. I. Pogson, unpublished observation).
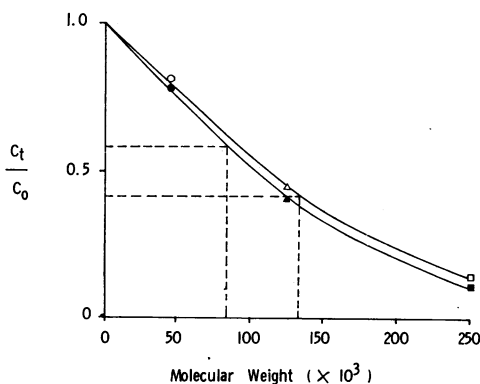
FIGURE 3 Typical partition cell distribution pattern obtained with undiluted extracts of acidotic (open symbols) and normal (closed symbols) rat kidney cortices. Details of practical procedures are given in the text. The molecular weights of the marker enzymes were assumed to be as given in the legend to Fig. 2. Dotted lines indicate C_t/C_0 ratios (ratio of enzyme activity in top compartment to that in the original extract) obtained for PEPCK activities in the two cases ○●, triosephosphate isomerase; △▲, lactate dehydrogenase; □■, catalase.

are close to those reported by Ballard and Hanson (14) for purified rat liver PEPCK, and by Chang and Lane (24) for the pig liver mitochondrial enzyme.

Since dilution of tissue extract is inherent to the sucrose gradient technique, it was considered that a method involving the use of undiluted extracts might reflect more accurately on the true in vivo state of PEPCK. Fig. 3 shows a typical distribution pattern obtained with the partition cell in the analytical ultracentrifuge. This method indicates molecular weight values of 83,000 and 128,000, respectively for the PEPCK's from normal and acidotic kidneys (values are the means of duplicate determinations).
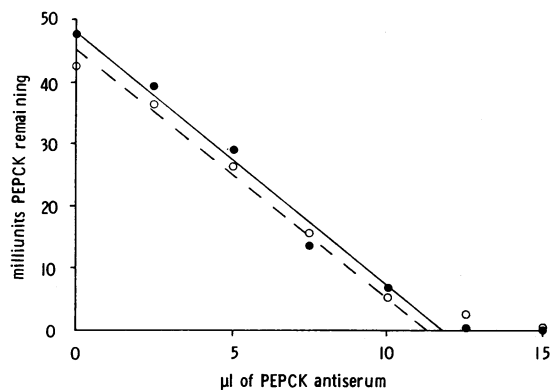

FIGURE 4 PEPCK-antibody titration of liver and kidney cortical extracts from normal rats. Details of practical procedures are given in the text. ○, kidney; ●, liver.
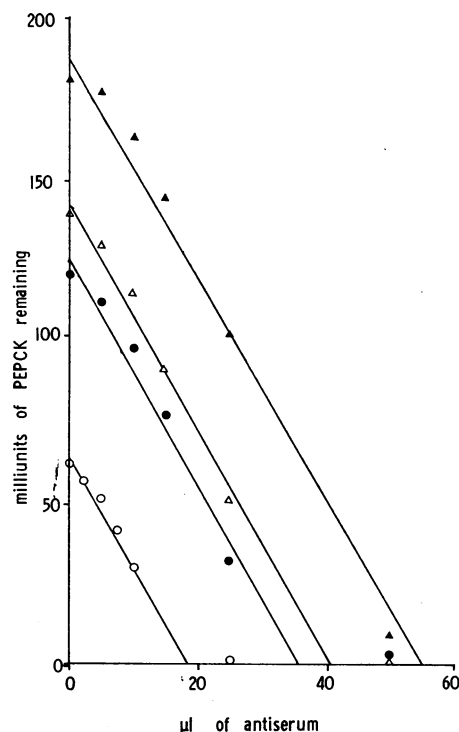
FIGURE 5 PEPCK-antibody titrations of kidney cortical extracts from variously-treated animals. Details of practical procedures are given in the text. ○, controls; ●, steroid-treated; △, acidotic; ▲, acidotic steroid-treated.

Antibody titrations. Antibody titration data for normal rat liver and kidney extracts are given in Fig. 4. PEPCK's for both sources react similarly with the purified antibody to rat liver PEPCK. The specific
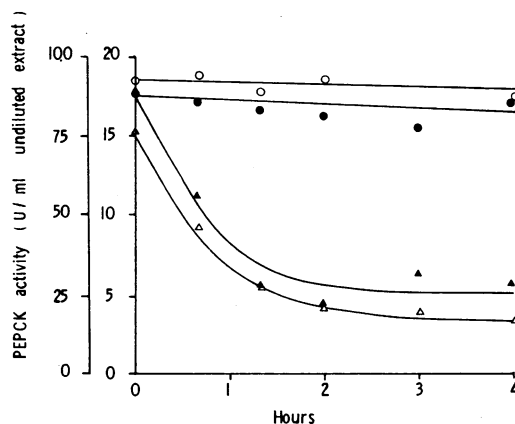

FIGURE 6 Stability of rat kidney cortex PEPCK. Details of practical procedures are given in the text. Open symbols represent values with extracts from acidotic animals (left-hand ordinate), closed symbols those from normal animals (right-hand ordinate). ○●, undiluted extracts; △▲, extracts diluted 1:25 with 50 mM Tris Cl pH 7.4.

activity of the antibody was 4.0 U PEPCK neutralized per ml of antiserum in both cases.

Fig. 5 shows similar titrations with extracts from the kidneys of normal, acidotic, steroid-treated, and acidotic steroid-treated animals. All titration curves are parallel indicating that changes in the total catalytic activity of PEPCK in the various conditions are associated with comparable changes in immunoreactivity.

Stability studies. The stability of PEPCK from acidotic kidneys was investigated by following enzymic activity in incubations at 20°C (Fig. 6). At a dilution of 1:25 activity rapidly declined to a new steady-state level; in the case of normal extracts this was 19%, and for acidotic extracts it was 33% of the original value. In undiluted extracts (i.e., with no buffer added) PEPCK activity remained at its original value for 4 hr. Similar curves were observed with extracts from normal kidneys.

The rate of decrease of PEPCK activity was markedly reduced (by approximately 90%) by the addition of 10 mM ATP, and to a lesser extent by ADP and AMP. Fluoride and EDTA were without effect on the rate of inactivation in both cases.

DISCUSSION

The observations reported in this paper lead to the following conclusions: (a) All PEPCK activity in rat kidney, however synthesized or induced, reacts identically with purified antibody under the conditions described. (b) The maximal stimulation of PEPCK induction by Steroid and NH₄Cl together is greater than the maximal stimulation elicited by either independently. (c) The increase in total PEPCK produced by acidosis in the steroid-treated rat is similar quantitatively to that produced in the normal animal by the same treatment, i.e., steroid- and acid-induced effects are additive, not synergistic. (d) Actinomycin D is effective in inhibiting the increase in PEPCK activity elicited by steroid, but not that resulting from acid treatment. (e) Acidosis is accompanied by an increase in the apparent molecular weight of kidney PEPCK.

It is difficult to reconcile these data with the "simplest" hypothesis, involving PEPCK induction through the actions of both steroid and acidosis at a single point in the sequence DNA→→→ messenger RNA→→ protein. For example the additive nature of the response in animals treated with saturating doses of both triamcinolone and ammonium chloride argues that the points of action of the two inducers may be distinct. Similarly the relative insensitivity to actinomycin D of the acidosis-induced rise in PEPCK in comparison

with that induced by steroid further serves to indicate different loci for the two effects.

In view of the similarity in antibody titers of extracts from acidotic, steroid-treated, and normal rats, it is apparent that the observed increases may be attributed to appearance of new *active* protein rather than to any direct or indirect effects on the kinetics or catalytic activity of the existing PEPCK. The increase in activity may theoretically arise through several possible mechanisms, e.g.: (a) *De novo* synthesis of protein involving increases in both messenger RNA transcription and translation. (b) *De novo* synthesis of protein involving an increase in messenger translation only. (c) Conversion of an inactive protein precursor into active enzyme. This might be (a) reversible, and possibly enzymically controlled or (b) irreversible, in a process analogous to that of zymogen activation. In this category one would also include the appearance of enzyme due to an increased rate of peptide release from the ribosome.

All of these processes may in addition be coupled to simultaneous effects upon the rate of degradation of pre-existing enzyme; changes in degradation *alone,* however, cannot result in increased enzyme titers.

The sensitivity of the triamcinolone-induced rise in kidney PEPCK to low levels of actinomycin D suggests that this steroid may act through mechanism (a). The insensitivity of the acidosis effect to similar doses of actinomycin D conversely suggests that mechanism (a) may not apply in this case. Previous results with ethionine as a blocker of messenger RNA translation (12) may be taken to indicate further that a mechanism similar to that listed under (c) could be involved in acidosis. In view of the known difficulties in the interpretation of experiments with protein synthesis inhibitors, however, it is difficult to be categorical as to the mechanisms involved on the basis of the results here presented. Further work on the direct elucidation of the rate-limiting steps involved in PEPCK induction is needed to clarify this point.

The presence of a higher molecular weight, presumably dimeric, species of kidney PEPCK in concentrated cytoplasm from ammonium chloride-treated rats is consistent with an effect of acidosis on degradation mediated through the interconversion of monomeric and dimeric species (27). Such an interconversion might well involve enzymically controlled phosphorylation or adenylylation reactions as suggested by Exton (25). The observation that adenine nucleotides aid in the stabilization of enzymic activity is further consistent with such an interpretation. The instability of the dimeric species towards dilution might similarly be explained by reductions in the concentrations of essential cofactors and/or by the relatively increased activity of hydrolytic

enzymes (phosphatases or adenylyltransferases) which do not require the presence of other small substrate molecules.

Foster, Lardy, Ray, and Johnston (26) have reported that the induction of PEPCK in liver occasioned by administration of tryptophan differs from that induced by hydrocortisone in its insensitivity to protein synthesis inhibitors. Although these workers noted differences in the short-term stability of the "two" PEPCK's in the absence of thiol, their observations are basically similar to those reported in the present paper.

In view of the discrepancies between oxaloacetate concentrations in isolated tissues with high gluconeogenic rates (28) and those necessary for significant rates with PEPCK in vitro (14, 24, 29), one may further conjecture that the binding of oxaloacetate to the dimeric species may be much tighter than that to the monomer. Resolution of this problem must await the development of convenient assay techniques applicable to concentrated crude extracts.

ACKNOWLEDGMENTS

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