LABILITY OF THE PROTEIN BIOSYNTHESIS SYSTEM OF THE MOUSE KIDNEY AND ITS RELATIONSHIP TO THE REGULATORY EFFECT OF ANDROGENS

By

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ABSTRACT

The amino acid (leucine, phenylalanine) incorporation system of the postmitochondrial fraction (S2) from the kidney of normal, castrated and testosterone propionate treated mice lost all of its activity on storage over-night at 5°C. The loss in activity was evident within one hour of storage at 4°C. Most of the amino acid incorporating activity was lost within 10 min on preincubation at 37°C. Addition of polyuridylic acid was not able to restore the activity. Neither castration nor the administration of androgen influenced the rate of loss of the amino acid incorporating activity. Separation of the soluble (S3) and microsomal (Ms) fractions and preincubation of either of the fractions produced loss in activity. The addition of poly U restored the activity of the preincubated Ms but not the preincubated S3 fraction. Storage of S2 at -20°C only retarded the rate of loss in activity. The addition of poly U to the enzyme system restored all of the phenylalanine incorporation activity for the castrated mice and most but not all of that of the androgen treated mice. The loss in activity was not accompanied by any detectable change in the RNA.

In a previous report (Kochakian et al. 1963) it was noted that the amino acid incorporating activity of the postmitochondrial fraction of mouse kidney was completely lost on storage overnight at 5°C. Lability of cell free preparations

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from mouse (Robinson & Novelli 1962) and rat (Sachs 1957; Shigeura & Gordon 1960) liver also has been reported but not studied in detail.

This report is concerned with a partial characterization of the labile factors and their possible relationship with the decrease in amino acid incorporating activity after castration and the increase after androgen stimulation.

MATERIAL AND METHODS

Mice

The mice originated from the Holtzman-Swiss strain and were raised in our colony. The litters were limited to eight pups per mother. The mice were kept in an air-conditioned room at 26 to 28°C with constant artificial light of 13 h per day. They were fed ad libitum Jim Dandy Dog Checkers (Western Grain Company, Birmingham). Castration was performed under ether anaesthesia.

Androgen

The testosterone propionate was implanted subcutaneously in the back as a cylindrical pellet of approximately 15 mg (Kochakian 1944). Testosterone* in aqueous suspension was injected subcutaneously.

Preparation of Tissue

Food was removed from the mice at 4:30 p.m. of the day preceding autopsy. The animals were killed by separating the spinal chord and bleeding from the blood vessels of the neck. The kidneys were removed and placed into 2 ml of ice-cold homogenizing medium in a round bottom homogenizing tube. Immediately after completion of the autopsies, the homogenizing medium was increased to 5 ml per gram of tissue and homogenization was performed by 9 to 11 strokes in 30 s of a Teflon pestle attached to an electric motor. The homogenizing tube was kept in an ice-water bath. The homogenates were centrifuged at 12 000 × g for 10 min in a refrigerated International Centrifuge (PR2) with high speed attachment and No. 296 head. The chamber temperature was 0 to −5°C. The supernatant fraction was used as the enzyme preparation. The microsomes were separated from the soluble fraction by centrifugation at 105 000 × g for one hour. The polysomes were prepared as previously described (Wettstein et al. 1963; Kochakian et al. 1969, in press).

Materials

The sodium adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Pabst Laboratories), phosphocreatine (PC) (Sigma), phosphocreatine kinase (PCK) (Sigma ≈ 31 μm units/mg at 30°C) and polyuridylic acid (poly U) potassium salt with S = 3.9 (Miles Laboratories) were used as purchased. They were dissolved in the homogenizing medium prior to use. The 14C-amino acids (California Corporation) were dissolved in water and sufficient 13C-L-amino acid (Nutritional Biochemicals or Sigma) was added to give the desired specific radioactivity. The solutions were stored in the deep freeze. The 14C-amino acids as purchased were: DL-leucine-1-14C, 12.0 μCi/μmole; L-phenyla-
lanine-1-\textsuperscript{14}C, 10.3 \(\mu\)Ci/\(\mu\)mole; L-phenylalanine-U-\textsuperscript{14}C, 10.4 \(\mu\)Ci/\(\mu\)mole. No difference was noted in the rate of incorporation into protein between the L- and DL-phenylalanine but the DL-form gave higher blank values. The Macaloid\textsuperscript{9} was extracted as described by Petermann (1964). The other chemicals were reagent grade.

The homogenizing medium was a modification (Kochakian et al. 1969, in press) of the previously used solutions (Kochakian et al. 1963). The concentration of Mg\textsuperscript{2+} and K\textsuperscript{+} was changed to provide optimum activity. The homogenizing medium contained: 0.25 mM sucrose, 7.5 mM magnesium acetate, 100 mM potassium chloride, 35 mM tris and 6 mM mercaptoethanol. The pH was adjusted to 7.7 by the addition of 1.0 n HCl.

The reaction mixture contained: 0.1 \(\mu\)mole (0.4 \(\mu\)Ci) L-aminio acid in 0.2 ml water; 1.0 \(\mu\)mole ATP, 0.2 \(\mu\)mole GTP and 20 \(\mu\)moles PC in 0.1 ml homogenizing medium; 40 \(\mu\)g PCK in 0.1 ml homogenizing medium; 0.3 ml homogenizing medium and 0.3 ml tissue preparation. When poly U or more enzyme was used, the amount of homogenizing medium was reduced so that the final volume of the reaction mixture was maintained at 1.0 ml. Other minor modifications are indicated in the legends. The incubation tubes were kept in an ice-water bath prior to addition of the tissue preparation. Incubation was for 30 min at 37\textdegree C. The reaction was terminated by the addition of 4 ml of ice-cold 0.4 n HCl\textsubscript{4}. The washing and plating of the protein and the extraction and determination of the ribonucleic acids were as previously described (Kochakian et al. 1963). The radioactivity of the protein was determined in a Nuclear-Chicago low background (2 cm\textsuperscript{2}) system of 25 per cent efficiency. At least 1000 counts per sample were obtained.

**RESULTS**

*Storage at 4\textdegree C*

Storage overnight of postmitochondrial fraction in a refrigerator set at approximately 5\textdegree C resulted in complete loss of activity by the next morning (Kochakian et al. 1963). The addition of reduced glutathione at 5, 10 and 20 \(\mu\)moles per ml of postmitochondrial fraction did not prevent the loss of activity in contrast to the protective effect reported for mouse liver stored at -20\textdegree C (Robinson & Novelli 1962). Therefore, storage in an ice water bath at shorter intervals was studied. A decrease in amino acid incorporating activity was evident within one hour and continued for the duration of the study so that after four hours, the preparation from the castrated mice had lost practically all of its activity (Fig. 1). The addition of polyuridylic acid to the phenylalanine system enhanced the incorporation activity but did not alter the rate of decrease during storage. Furthermore, the administration of androgen to the mice stimulated the expected (Kochakian et al. 1963) increase in the rate of incorporation of both phenylalanine and leucine but did not change the rate of loss in activity. The losses were in parallel with those in the postmitochondrial fraction from the castrated mice. Practically identical results were obtained in another series of experiments.

\textsuperscript{9} The Macaloid was provided by the Baroid Division, National Lead Co., Houston, Texas.
Loss of amino acid incorporating activity by mouse kidney postmitochondrial fraction on storage in an ice-water bath. The mice were castrated at one month of age. The testosterone propionate (TP) pellet (15 mg) was implanted subcutaneously four months later and all the mice were killed 5 days later. TP absorbed was 1.2 mg. There were nine control and five treated mice. The average body and kidney weights at autopsy were: Normal 37 (34-40) g, 380 (300-480) mg; TP 38 (36-39) g, 534 (500-595) mg. The samples were stored in an ice-water bath. The L-phenylalanine-U-14C (177 μCi/μmole) and L-leucine-U-14C (200 μCi/μmole) were added at 0.4 μCi/reaction. Identical results were obtained when 0.8 μCi DL-leucine (4 μCi/μmole) and DL-phenylalanine (4 μCi/μmole) were used instead of the uniformly labelled amino acids except that the absolute radioactive counts in the samples were lower (cf. Fig. 2).

The extension of the time of homogenization from 30 to 120 s resulted in a 25% loss of activity but did not influence the rate of loss on storage in an ice water bath. The type of homogenizer did not make any difference. Similar results were obtained when the homogenization was made with a Potter-Elve-hjem type glass tube and Teflon pestle or an all glass tissue grinder (Kontes Glass Co., No. 88545C).

Diatomaceous earth e.g. bentonite (Petermann 1964) is reported to adsorb RNase and prevent its action. The addition of Macaloid (Richards et al. 1963) 2 mg/ml, prior to centrifugation of the homogenate at 12 000 × g, had no effect on the loss in activity (Fig. 2). An increase to 5 mg/ml also was ineffective and in addition, resulted in a loss of total activity with a concomitant loss in RNA. The RNA apparently was adsorbed on the Macaloid and settled with the nuclear fraction on centrifugation (Petermann 1964).

Storage did not change the RNA content of the fractions in the above experiments.
The failure of Macaloid to stabilize the amino acid incorporating activity of the post-mitochondrial fraction of the normal mouse kidney. The mice were eight months old. The average body weight was 42 (41–46) g and the average kidney weight 574 (470–685) mg. The procedure was as described in the text. The Macaloid at 2 and 5 mg/ml was added after homogenization, and mixed with the homogenate before centrifugation at 12,000 × g for 10 min. The supernatant fractions were stored in an ice-water bath and aliquots in duplicate were removed for assay at the indicated times. The protein of the S 12,000 × g fraction aliquots (0.3 ml) was: control 2.9, Macaloid (2 mg/ml) 2.4 and (5 mg/ml) 2.0 mg; the RNA of the respective aliquots was 181, 104, 77 µg. The DL-leucine-1-14C was made to 4 µci/µmole/ml; 0.2 ml was added to each reaction. Incubation was for 30 min.

**Preincubation at 37°C**

The rapid lability at ice water temperature, prompted the determination of the degree and rate of lability at the enzyme reaction temperature. The enzyme activity was almost completely lost after 15 min, and very little activity remained after 30 min of preincubation of the postmitochondrial fraction prior to addition to the incorporating system (Fig. 3). The addition of poly U was not able to restore the enzyme activity. In another series of experiments without preincubation, additional poly U was added 15, 30 and 60 min after incubation without any further increase in phenylalanine incorporation.

**Intracellular localization of the labile factors**

The postmitochondrial fraction was separated into the soluble (S₃) and microsomal (Ms) fractions by centrifugation at 105,000 × g for one hour. Each fraction was divided into two portions; one for storage at 4°C and the other for
Loss of amino acid incorporating activity by preincubation at 37°C of the postmitochondrial fraction of the mouse kidney. The five mice were 5 to 6 months old. The average body and kidney weights were 32 (31–33) g and 525 (465–605) mg. The postmitochondrial fractions were pooled and a portion placed in the water bath at 37°C. Aliquots were removed for assay after the indicated time periods. The DL-phenylalanine (8 μCi/μmole) was added at 0.8 μCi per reaction. Incubation was for 30 min.

Preincubation. Preincubation of either fraction resulted in identical loss in amino acid incorporation activity (Fig. 4). The addition of poly U to the enzyme system restored the activity when the microsomal fraction, but not the S₃, was preincubated. The treatment of the mice for seven days with a subcutaneously implanted pellet of testosterone propionate stimulated the expected enhancement of phenylalanine incorporation but did not alter the effect of preincubation on either the S₃ or Ms fractions. Identical results were obtained in a separate experiment with normal mice.

Storage of postmitochondrial fraction at -20°C

The postmitochondrial fractions from four sets of experiments were placed in the deep freeze (-20°C) immediately after assay for amino acid incorporating activity. The samples were reassayed 10 and 19 days later (Fig. 5). The amino acid incorporating activity had markedly decreased after 10 days with no further loss after another 9 days of storage. The preparation from the mice which had been treated with testosterone propionate for 48 h lost as much absolute activity as the other preparations after 10 days and decreased further after the additional 9 days of storage to the same minimal level as the other preparations. The addition of poly U to the reaction mixture restored the activity of the preparation from the castrated mice and also from the mice which received an ineffective androgen stimulation (T-18). The activity of the S₂ fractions from the mice which had an effective stimulation by the androgen...
Loss of amino acid incorporating activity by preincubating separately the microsomal and soluble fractions of the postmitochondrial fraction of mouse kidney and the influence of poly U. The mice were castrated at one month of age. The pellet of testosterone propionate (TP) was implanted three months later. All of the mice were killed after seven days. There were 12 castrated controls and 8 treated mice. The amount of TP absorbed was 1.5 mg. The body and kidney weights at autopsy were: C = 29 (27–30) g, 276 (265–325) mg and TP = 32 (29–32) g, 465 (440–500) mg. The DL-phenylalanine-1-14C (8 μCi/μmole) was added at 0.8 μCi per reaction. Incubation was for 30 min. The preincubated fraction is designated for each curve.

Fig. 4.

treatments was restored nearly but not completely to the values at zero time. Confirmatory results were obtained in another four sets of separate experiments with storage periods of 4, 10, 13 and 19 days*.

The perchloric acid precipitable RNA in the above experiments was not detectably changed by the storage at –20°C.

The loss in both leucine and phenylalanine incorporating activity was appa-

* The four sets of experiments were:
a) Normal, castrated and castrated mice implanted subcutaneously with a testosterone propionate pellet for four days.
b) Controls and castrated mice treated with a testosterone propionate pellet for 7 days. Storage was for 13 days.
c) Controls and castrated mice killed 12, 18, 24, 30, 36 and 42 h after the subcutaneous injection of 2.5 mg of testosterone (aqueous suspension). Storage was for 10 days.
d) Controls and castrated mice autopsied 18 and 24 h after the subcutaneous injection of 2.5 mg of testosterone (aqueous solution). Storage was for 19 days.
Lability of the amino acid incorporating system of the mouse kidney postmitochondrial fraction on storage at -20°C. The experiments were performed eight months later. The 0.05 ml of testosterone (aqueous suspension, 50 mg/ml) was injected subcutaneously 18 (T-18), 24 (T-24) and 48 (T-48) h before autopsy. There were four mice per group. The average body weights were 44 (42-48) g. The kidney weights were: Control 400 (380-430) mg; T-18: 419 (370-445) mg; T-24: 413 (400-425) mg and T-48: 464 (435-505) mg. The DL-phenylalanine-1-14C (8 μCi/μmole) was added at 0.8 μCi per reaction. Incubation was for 30 min.

rent within 24 h of storage at -20°C (Table 1). The addition of poly U partially restored the lost activity. The results were essentially the same for the three groups of mice. In three more sets of experiments, practically identical results were obtained in two of the experiments but the other experiment showed no significant decrease in activity. The inability to restore all of the activity by the addition of poly U was due to loss of factor(s) in the S₃ fraction. Storage of this fraction for 24 h resulted in an approximately 20 per cent decrease in the incorporation of leucine into protein by polysomes (Fig. 6). A small further loss occurred after another 24 h of storage but no further loss after 7 days. The microsomal fraction maintained its activity.

**DISCUSSION**

The amino acid incorporating system of the mouse kidney and also liver (Robinson & Novelli 1962) apparently is more labile than similar systems in the tissues of other species. The system of the liver, kidney, seminal vesicles and
prostates of the guinea pig is much more stable. The postmitochondrial fractions of these tissues did not lose detectable amounts of activity on storage for as long as 6 h in an ice water bath and were less sensitive than the mouse kidney preparation on preincubation at 37°C (unpublished). Lability of the amino acid incorporating system of rat liver also has been noted (Sachs 1957; Shigeura & Gordon 1962) but has not been studied in detail. The lyophilization of the postmitochondrial fraction, however, "yielded a dry protein which could be stored at −20°C for several months without any appreciable loss of activity« (Sachs 1957).

The lability of the enzyme system is due to at least two factors located in different parts of the cell. One is very likely a RNA hydrolyse which acts on the messenger RNA to split the polysomes into smaller units. This effect could be due to a particular RNase (Blobel & Potter 1966; Lawford et al. 1966) or that the messenger RNA's of the mouse tissues are not protected from the action of the enzyme due to exposure of hydrolytic sites because of lack of protein coating or structural conformation. Another possibility is an insufficiency of endogenous RNase inhibitors (Roth 1958). In any event, the restoration of phenylalanine incorporating activity after preincubation of the microsomal fraction indicates that the breakdown of the polysomes initially did not proceed beyond the monosomal stage.

Storage of the postmitochondrial fraction at −20°C retarded the rate of loss

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**Table 1.**

Loss in amino acid incorporation activity by the postmitochondrial fraction of mouse kidney after storage at −20°C for 24 hours.

<table>
<thead>
<tr>
<th>Storage at −20°C</th>
<th>Normal</th>
<th>Castrate</th>
<th>Castrate and TP</th>
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<tr>
<td>h</td>
<td>Leu</td>
<td>Phe</td>
<td>Leu</td>
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<tr>
<td>0</td>
<td>843</td>
<td>402</td>
<td>563</td>
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<tr>
<td>24</td>
<td>714</td>
<td>322</td>
<td>512</td>
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<tr>
<td>Diff. %</td>
<td>−15</td>
<td>−19</td>
<td>−9</td>
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* The mice were castrated at one month old. The experiments were performed one month later. The testosterone propionate (TP) was implanted subcutaneously as a 15 mg pellet, seven days before autopsy; the average absorption was 1.1 mg per mouse. There were 15 normal, 21 castrated and 9 TP treated mice. The kidney weights were: Normal 488 (405–525) mg, Castrate 361 (275–425) mg and TP 509 (435–600) mg. The L-leucine-U-14C and L-phenylalanine-U-14C (20 µCi/µmole) were added at 1 µCi per reaction. The rest of the procedure was as described in the test.
Stability of $S_3$ fraction of mouse kidney on storage at $-20^\circ$C. The mice were three to four months old. The average of the body weights was 42.6 (34-49) g and of the kidney weights 614 (440-725) mg. The $S_3$ fractions from the kidneys of forty-five mice were pooled and five ml aliquots were stored at $-20^\circ$C for use at the indicated times. The polysomes were prepared as previously described (Wettstein et al. 1963; Kochakian et al., in press). The reaction mixture contained the indicated amounts of $S_3$, polysomes containing 95 $\mu$g RNA and 1.0 $\mu$Ci L-leucine-U-14C (20 $\mu$Ci/umole). The rest of the reactants and procedure were as described in the text.

of activity. The stabilization was more effective for the microsomal than the soluble factors of the amino acid incorporating system. The inability of poly U to restore full activity of the postmitochondrial fraction after prolonged storage at $-20^\circ$C indicates the loss of factor(s) in addition to messenger RNA. Storage of the supernatant fraction resulted in an initial small loss in activity. This labile factor could be transferase II which is reported to be very labile (Gasior & Moldave 1965) or some other substance(s) (Hoagland 1961).

It is of particular significance that the inability to restore full activity by the addition of poly U occurred only for the postmitochondrial fraction from the kidneys of mice which received a stimulating dose of testosterone. Thus, part of the stimulating effect of androgens on protein biosynthesis may be through factors in the soluble fraction. One of the factors in the protein biosynthesis system is regulated by androgens. The polysomes and also the monosomes of the mouse kidney decrease after castration and increase on androgen stimulation (Kochakian et al. 1969, in press). One of the factors in the soluble
fraction, the amino acid activating enzyme, changes in activity in direct proportion with the changes in kidney weight after castration and androgen administration (Kochakian et al. 1963).

These studies emphasize the importance of careful consideration of the different components (known and unknown) of a multiple biological system like the amino acid incorporating system in the interpretation of hormonal (or other) regulation.

REFERENCES


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