Disappearance of Radioactivity from the Various Ribonucleic Acid Pools and Acid-Soluble Fractions of Mouse Liver and Kidney after a Single Injection of Labelled Orotic Acid

THE EFFECT OF CASTRATION

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1. An attempt was made to study the rate of synthesis as well as the distribution of RNA in the various cellular fractions in the livers and kidneys of normal and castrated mice. 2. The tissue was fractionated by the procedure of Blobel & Potter (1967). By using this method it was not possible to find any pronounced difference in the relative proportions of RNA in isolated subcellular fractions when kidneys from normal and castrated mice were compared. On the other hand there was an indication of a shift toward the bound ribosomes in livers from normal mice in comparison with livers from castrated mice. 3. Disappearance of the radioactivity followed the pattern of the first-order reaction. Comparing the half-lives of RNA in liver and kidneys it was found that in the latter in both groups of animals half-lives were shorter no matter which cellular fraction was studied. 4. The half-lives for total homogenate RNA, total ribosomal RNA and low-molecular-weight RNA from kidneys of castrated mice were approximately 20-25% longer than the half-lives for the corresponding fractions from normal mouse kidneys. 5. An explanation is put forward for the anomalous finding that RNA from the castrated-mouse kidneys has a higher specific radioactivity than that isolated from normal mice.

The study of RNA turnover in the kidney is beset by complications found with other organs plus some additional problems generated by the rôle of the kidney in the conservation of constant concentration of blood constituents. Also, there is a marked heterogeneity of cell types with drastically different metabolic rôles in the kidney. Hormones, like androgens, might affect various cell types in a different way so many precautions are necessary in measurements of rates of synthesis of any given molecular species when expressed for an organ as such.

I have chosen the method in which the decline in radioactivity is followed after intraperitoneal administration of a single 'pulse' of labelled substrate. This method has the major disadvantage that during the 'pulse' the label incorporated into a given macromolecular species is inversely propor-

tional to the half-life of that species. Further, hormones might affect the resorption of the labelled compound from the site of injection and subsequently change the availability of isotope for different organs and tissues. This is especially disadvantageous when turnover rates of most labile RNA species are in question, because the changes in the transport of label from the injection site to the site of incorporation in the liver and kidney might create false impressions. Earlier investigators (Campbell, Serck-Hansen & Lowe, 1965; Commarano, Guidice & Lukes, 1965; Loeb, Howell & Tomkins, 1967; Webb, Blobel & Potter, 1964; Webb, Blobel, Potter & Morris, 1965; Talal & Kaltreider, 1968; Priestly & Malt, 1969; Tata, 1967) have attempted to study the ribosome-membrane interaction in terms of the distribution between free and bound ribosomes under a wide variety of conditions. Using the newer methodology of Blobel & Potter (1966, 1967) I have investigated the distribution as well as the disappearance of the

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radioactivity in these components in the liver and the kidney of normal and castrated mice. The experiments in the present paper show that, although there was a decrease in total cellular RNA and ribosomal RNA in the livers and kidneys of castrated mice, there is no striking difference in relative proportions of free and bound ribosomes. This is indicated both morphologically (T. Hirone, M. Nishida & C. D. Kochakian, unpublished work; Tessmann, 1968) and chemically. The results also indicate that there is no substantial change in the rate of disappearance of the radioactivity from different RNA pools after castration. The results do not support, therefore, the previously suggested interpretation of the [^{14}C]orotic acid incorporation into RNA and acid-soluble pool of mouse kidney (Kochakian & Hill, 1966).

METHODS AND MATERIALS

Experiments were performed on albino male mice purchased from Charles River Breeding Laboratories, North Wilmington, Mass., U.S.A. At the age of 40 days one group of mice was castrated. All the mice were killed at the indicated times 60 days after castration. [$6\text{-}^{14}\text{C}$]Orotic acid (sp. radioactivity 34 mCi/mmol) was obtained from Centre d'Energie Atomique, Département des Radioéléments, B.P. no. 8, 91-Gif-sur-Yvette, France.

Tissue fractionation. Animals were injected intraperitoneally with $3\mu\text{Ci}$ of [$6\text{-}^{14}\text{C}$]orotic acid, and then killed by bleeding at the following time intervals: 1, 3, 6, 12 and 24 h, 2, 3, and 15 days. The method used for fractionation of the whole liver and kidney homogenates into subcellular components was essentially that of Blobel & Potter (1967). Their original method was used with only slight modifications. Instead of centrifuging the nuclei from the kidney for 30 min they were centrifuged for 45 min, because preliminary experiments indicated that shorter centrifugation times resulted in lower recoveries. The next modification involved the measurement of low-molecular-weight RNA. Contaminating Triton X-100 was not removed by careful washing of the precipitated low-molecular-weight RNA. Therefore, after hydrolysis in alkali there was still a substantial amount of Triton X-100 present as determined by spectral analysis in a Cary 14 spectrophotometer. It seems that Triton X-100 binds rather strongly to RNA and protein in acid conditions, whereas under alkaline conditions it remains free in solution. Due to its very high extinction coefficient, even (see Fig. 1) minute amounts of Triton X-100 interfere with u.v. measurement of the hydrolysed RNA. Therefore the orcinol method (Ceriotti, 1955) was used to determine the amount of low-molecular-weight RNA.

Each point on the graphs represents the average values from two or three mice. The kidneys from each group of mice were pooled whereas livers were processed individually. RNA fractions from the liver and kidneys in both castrated and normal groups of mice were checked for purity in a sucrose gradient by using the sodium dodecyl sulphate method of Kurland (1960). On the basis of previously established criteria, the fractions contained (total ribosome and free ribosomes) 28 and 18S RNA,

Fig. 1. U.v.-absorption spectra of (—) pure Triton X-100 in water, (---) alkali-hydrolysed low-molecular-weight RNA precipitated from the medium containing Triton X-100, (—) alkali-hydrolysed low-molecular-weight RNA precipitated from the medium free of Triton X-100. Spectra were obtained on a Cary 14 recording spectrophotometer. Samples were read against water.

whereas low-molecular-weight RNA travelled at an average speed of 4–6S.

Preparation of the post-mitochondrial supernatant and RNA extraction. A post-mitochondrial supernatant (S_2) was prepared by centrifuging the homogenate in a Spinco L2-50 ultracentrifuge at $12000g_{av}$ for 10 min. From the combined nuclear-mitochondrial pellet RNA was extracted by the procedure described by Penman (1966). Cytoplasmic RNA was isolated from the post-mitochondrial supernatant by the addition of 10% (w/v) sodium dodecyl sulphate to a final concentration of 1.0% followed by the addition of an equal volume of water-saturated phenol. The solution was shaken for 30 min at 25°C , and the aqueous layer was separated by low-speed centrifugation. The aqueous layer was re-extracted twice by the addition of equal volumes of water-saturated phenol. RNA was precipitated from the aqueous phase (made 2.0% with respect to potassium acetate) by the addition of 2.5 vol. of cold ethanol, removed by centrifugation, and washed several times with cold 75% (v/v) ethanol. The labelling pattern in this purified RNA was checked on a linear 5–20% (w/v) sucrose gradient containing 0.1M-NaCl–0.01M-sodium acetate buffer, pH 5.2.

Determination of RNA and DNA. RNA was determined by the method described by Munro & Fleck (1966), DNA was determined according to Burton (1956), as modified by Giles & Meyers (1965).

RESULTS

Distribution of RNA in various fractions from mouse liver and kidney homogenates. The distribution of RNA in homogenates of liver and kidneys from normal and castrated mice is presented in Table 1. Castration produced a significant decrease in the total RNA/DNA ratios in both kidney and liver. Further fractionations of the tissues were conducted to determine whether the results indicated in Table 1 for total RNA might be due to a redistribution of ribosomal RNA. Table 1 presents summarized data from 18 experiments in which the distribution of hepatic and kidney RNA of the whole tissue homogenate was assayed. In the kidney of castrated mice there is no statistically significant change in nuclear RNA, but there is a pronounced decrease in total ribosomal RNA, low-molecular-weight RNA and bound ribosomal RNA. The comparison of the relationship of free to bound ribosomes in normal and castrated-mouse kidneys reveals that there is no shift under the described experimental conditions. The amount of bound ribosomes in our experiments as a percentage of total ribosomes (50.6–50.7%) or total homogenate RNA (39.7–38.5%) is substantially above the values reported by Priestly & Malt (1969) for mouse kidney. The reason for this discrepancy may be due to different methods of tissue fractionation, or a different method of tissue homogenization. In the livers of castrated mice, RNA was distributed in a somewhat different fashion from that in kidneys. Whereas there was a measurable decrease in the total homogenate as well as in the total ribosomal RNA fraction, there was no change in the fraction of free ribosomal RNA and low-molecular-weight RNA. Also, there were no differences in the amounts of nuclear RNA in the livers between normal and castrated mice. Although it is difficult

to conclude anything on just the basis of these observations, there is an indication of a shift toward free ribosomes in the livers from castrated mice. The amounts of RNA recovered from the nuclear fraction varied much more than in the other fractions which could possibly account for the lack of statistically significant differences. RNA labelled with [6-¹⁴C]orotic acid was extracted from the livers and kidneys of normal and castrated mice to ascertain whether castration qualitatively altered the RNA labelling pattern. RNA was extracted from the postmitochondrial supernatant of the liver and kidney homogenates and from the combined mitochondrial and nuclear pellet. The RNA from the nuclear fraction of the normal liver and kidney showed a typical short-time pulse-labelling pattern (Georgiev, 1967; Penman, Smith & Holtzman, 1966) with RNA of heterogeneous molecular weight being labelled. The cytoplasmic RNA from castrated-mouse liver and kidney showed the typical pattern obtained from the pulse-labelling *in vivo* (Henshaw, Revel & Hiatt, 1965). There was considerable labelling of the soluble RNA fraction and some labelling of the 18S and 28S fractions. The radioactive labelling pattern of the cytoplasmic RNA from the kidney of castrated mice showed no qualitative difference from that from normal mice. It is, therefore, concluded that castration does not, in any significant way, alter the types of RNA synthesized in the liver and kidney. However, castration significantly increased orotic acid incorporation into kidneys.

Disappearance of the radioactivity from RNA of various fractions of mouse kidney and liver. Besides studying the distribution of the RNA in various cellular fractions of the mouse kidney and liver, I also studied the influence of castration on the amounts of acid-soluble and acid-insoluble radioactivity as a function of time after a single injection

Table 1. *Distribution of RNA in the various fractions of mouse kidney and liver total homogenate*

All results are expressed as μg of RNA/mg of DNA. The average DNA content/cell in the mouse kidney nucleus was 5 pg. n.s. = Not significant.

	Total homogenate RNA	Nuclear RNA	Total ribosomes	Free ribosomes	Bound ribosomes	Low-mol.-wt. RNA*
Normal kidney	1930 $\pm$ 106	82.5 $\pm$ 7	1510 $\pm$ 129	745 $\pm$ 21	765 $\pm$ 89	237 $\pm$ 13
Castrated kidney	1475 $\pm$ 69	67.2 $\pm$ 6	1115 $\pm$ 76	548 $\pm$ 20	567 $\pm$ 43	197 $\pm$ 8
Level of significance	$t = 3.98$ $P < 0.001$	$t = 1.716$ $P < 0.1(\text{n.s.})$	$t = 2.85$ $P < 0.01$	$t = 4.34$ $P < 0.001$	$t = 3.14$ $P < 0.01$	$t = 2.4$ $0.02 > P < 0.05$
Normal liver	4253 $\pm$ 251	124.7 $\pm$ 10	3430 $\pm$ 151	740 $\pm$ 26	2690 $\pm$ 134	680 $\pm$ 20
Castrated liver	3545 $\pm$ 197	118.5 $\pm$ 8	2820 $\pm$ 151	730 $\pm$ 29	2090 $\pm$ 131	672 $\pm$ 31
Level of significance	$t = 2.215$ $P < 0.05$	$t = 0.495$ $P < 0.7(\text{n.s.})$	$t = 3.51$ $P < 0.01$	$t = 0.027$ $P < 0.1(\text{n.s.})$	$t = 3.96$ $P < 0.001$	$t = 0.03$ $P < 0.15(\text{n.s.})$

* This value was obtained by subtracting the free from total ribosomes.

Fig. 2. Incorporation of $[6\text{-}^{14}\text{C}]$ orotic acid into nuclear (Δ , $\circ$) and ribosomal (∇ , $\bullet$) RNA fractions isolated from normal ($\circ$, $\bullet$) and castrated (Δ , ∇) mice. An early incorporation period is represented. Fractions were isolated from (a) liver and (b) kidney. For the details see the Methods and Materials section.

of $[6\text{-}^{14}\text{C}]$ orotic acid. Since expressing data on the basis of mg of DNA, i.e. per cell, did not give substantially different results from specific radioactivity data (i.e. d.p.m./mg of RNA), I decided to use this last method in presenting our results on disappearance of the radioactivity from RNA. However, in the case of the acid-soluble fraction the results were expressed on the basis of mg of DNA, because this fraction was not further fractionated and, therefore, we have not obtained the values for specific radioactivities of different pyrimidine precursors. Figs. 2(a) and 2(b) present the results of the early incorporation periods, up to 72h, for the liver and kidney respectively. From the curves in Fig. 2 it is evident that the specific radioactivity of the liver nuclear RNA is consistently higher in normal mice than in castrated mice. The same is valid for the total ribosome fraction. Specific radioactivities of nuclear and ribosomal RNA were equal after 54h (crossover point) in livers of normal mice and after 45h in livers of castrated mice. The specific radioactivities for both RNA fractions from the kidneys of castrated mice are much higher than from normal animals. It is also noteworthy that specific radioactivities from corresponding liver fractions are much lower than those for the kidney from the same animals. In the case of mouse kidney, crossover points are almost identical in the fractions from normal and castrated mice, and they appear at 21h after the intraperitoneal injection of $[6\text{-}^{14}\text{C}]$ orotic acid (see Fig. 2b).

Fig. 3 shows the results pertaining to the acid-soluble fractions obtained from the liver and

Fig. 3. Disappearance of the radioactivity from the acid-soluble fraction of kidneys ($\bullet$, ∇) and livers ($\blacktriangle$, $\circ$) of both normal (∇ , $\blacktriangle$) and castrated ($\circ$, $\bullet$) groups of mice.

kidneys of both normal and castrated mice. Radioactivity is expressed on the basis of DNA content in the homogenate. The pattern of disappearance is similar for both organs and groups studied. It appears that all the curves presented may be resolved into two components, the first fast and the

second much slower, thus indicating that the disappearance of radioactivity follows first-order reaction kinetics. Radioactivity disappears slightly faster from the acid-soluble fraction in the kidney than in the liver. The amount of radioactivity per mg of DNA is slightly higher in the acid-soluble fraction of normal mouse liver than of castrated-mouse liver. This is opposite for the kidneys where there is more radioactivity in the acid-soluble fraction from the castrated mice. The half-life for the loss of radioactivity from the acid-soluble fraction is 134.5h for the normal mouse liver and 130h for the castrated-mouse liver. Half-lives in the kidney acid-soluble fractions are 115h and 120h for the castrated and normal mouse respectively.

From a comparison of the amounts of radioactivity per mg of DNA in the kidney and liver it is evident that there is more radioactivity accumulated

in the kidney on the DNA basis. In the case of kidney we may assume that this radioactivity represents the amount accumulated per cell because the mouse kidney nuclei are predominantly diploid (Palkovits & Fischer, 1968b). The situation with the liver is far less clear because cells in this organ have nuclei with different DNA contents varying from diploid to 16-ploid (Palkovits & Fischer, 1968a). So, we may assume that for kidney the amount of DNA per organ is roughly proportional to the number of the cells, whereas in the liver this might not be the case. The difference in the amounts of radioactivity is much more pronounced in the early stages after the injection and much less at the end of experimental period.

Fig. 4 summarizes further results on the specific radioactivity of the various RNA fractions in the subcellular components of livers from normal and castrated mice. Results are expressed as d.p.m./mg

Fig. 4. Summarized presentation of the disappearance of the radioactivity from RNA isolated from various cellular fractions of normal and castrated mice. $\circ$, Total ribosomal RNA; $\blacksquare$, total homogenate RNA; ∇ , nuclear RNA; $\bullet$, low-molecular-weight RNA; (—) represents the fast component of the nuclear RNA decay curve. (a) Normal mouse liver; (b) castrated-mouse liver; (c) normal mouse kidney; (d) castrated-mouse kidney.

of RNA and show that there is no substantial difference between the specific radioactivities of the normal and castrated-mouse liver. However, all the RNA fractions from normal livers contain more radioactivity per mg of DNA than those from castrated mice. The results indicate that the radioactivity from nuclear RNA declines after the first 2–3 days according to first-order kinetics. This curve can be easily resolved into two components, an initial fast section followed by a slower portion. The fast components have half-lives of 14.4 and 14.6 h for normal and castrated-mouse liver nuclear RNA respectively. The slow components have half-lives of 148 and 144 h in livers of normal and castrated animals respectively. From the second and third day, total homogenate, ribosomal RNA and low-molecular-weight RNA also follow first-order kinetics. Specific radioactivity of total ribosomal RNA is always higher than that of total homogenate, low-molecular-weight RNA and nuclear RNA in both groups of animals. Specific radioactivities of total ribosomal and whole homogenate RNA decline with half-lives of 148 and 130 h in livers of normal and castrated mice respectively. The specific radioactivity of low-molecular-weight RNA decreases in both groups of animals with a shorter half-life (124 h) in both normal and castrated mice. In summation, radioactivity in all the fractions from normal and castrated mice follows first-order kinetics and does not deviate from this pattern to the end of the experimental period. Although castration resulted in a significantly lower incorporation per mg of DNA, specific radioactivities of all the fractions studied from these two groups of animals are almost identical. The rate of disappearance of radioactivity is also very similar in these groups of mice, the only exception being the rate of disappearance in total homogenate and total ribosomal RNA.

Figs. 4(c) and 4(d) present the specific radioactivities of RNA in the various fractions from normal and castrated-mouse kidney homogenate. It is evident that in this organ the loss of radioactivity after the first 2–3 days also follows first-order kinetics. In all cases the specific radioactivity of the RNA fractions from the kidney homogenate exceeded that of the corresponding fractions isolated from the liver of the same mouse by a large amount. Values for the amount of radioactivity expressed as d.p.m. in RNA/mg of DNA are also higher in the mouse kidney than in the liver. With kidney nuclear RNA, as in the case of the liver, the disappearance curve can be separated into two components, one fast and one slow. The fast component has a half-life of 14.4 h in the normal mouse kidney and 12.5 h in that of the castrated animal. The half-life of the slow component also differs slightly in these two groups of animals, being

129 and 120 h in kidney of normal and castrated mice respectively. These values are substantially lower in comparison with the slow components of mouse liver nuclear RNA. Total ribosomal RNA and total homogenate RNA from normal mouse kidney have radioactive half-lives of 96 and 101 h respectively. In comparison with these values the corresponding fractions from castrated mice have radioactive half-lives of 122 h. There is a pronounced difference between the values for low-molecular-weight RNA fractions. The normal mouse kidney has a half-life for this fraction of 87 h whereas in the castrated-mouse kidney the value for this RNA is 104 h. This is the only pronounced difference between these groups of animals and it certainly deserves a thorough further investigation.

The values for the half-lives in all these fractions might be utilized to calculate the rate of synthesis of RNA in all of the various fractions of tissue homogenate. I think that this is still insufficient because the data for the immediate RNA precursors (i.e. nucleotides UTP and CTP) were not obtained in this experiment. Therefore, conclusions pertinent to the RNA synthesis derived solely on the basis of the present data might be very erroneous.

DISCUSSION

Androgen hormone influences a great number of metabolic activities in the cells (Commarrano *et al.* 1965). Among these activities is the stimulation of protein-synthesizing ability of mouse kidney cells, as has been demonstrated both *in vivo* (Novak, 1957; Kochakian, Hill & Aounuma, 1963) and *in vitro* (Frieden & Cohen, 1958; Frieden, Laby, Bates & Layman, 1957). Androgen hormones also stimulate the synthesis of RNA in kidney and liver of both castrated and normal mice (Kassenaar, Kouwnehoven & Querido, 1962; Kochakian & Harrison, 1962). The synthesis of all types of RNA in the mouse kidney is stimulated by treatment with male sex hormones (Kochakian, 1966). We have reported (Avdalović & Kochakian, 1969) that castration results in a sharp decrease in the RNA polymerase activity in isolated mouse kidney nuclei. An increase of almost 100% is observed in the Mg^{2+} -stimulated RNA polymerase activity 2 h after testosterone administration to castrated mice. At present, this appears to be the earliest measurable effect of testosterone on the mouse kidney.

In the present work I was primarily concerned with the measurement of the extent of incorporation of radioactive orotic acid into the various RNA fractions in livers and kidneys of normal and castrated mice. Since the accumulation of RNA in the cells might be a result of either decreased degradation or increased synthesis or both, I have tried to correlate the rates of incorporation of the

radioactive precursor with the rates of RNA synthesis. In the present set of experiments castrated and normal animals were used. Two months after castration and at the age of 3.5 months both groups of animals were in a steady state or at least in a state of growth that proceeded at a very slow rate. I was particularly interested in this state of animal's life because only under the conditions of steady state does the phenomenon of turnover have a proper meaning. As is evident from the results, castrated animals accumulated much more radioactivity in the kidney than normal ones, although they received the same amount of radioactivity per gram of body weight as normal intact mice. This unexpected effect was previously observed and described by Kochakian & Hill (1966). They interpreted their data as indicating a faster turnover of the acid-soluble RNA pool in the castrated mice.

The question has been raised as to what extent the rates of incorporation of radioactive precursor into any given macromolecule represent the actual rate of synthesis. This is especially important when orotic acid is used as a precursor in RNA synthesis. Normally, in the body, orotic acid is not present in a free form or as a long-lived intermediate. It exists only as a short-lived intermediate in the pyrimidine pathway (Hurlbert & Potter, 1952). There does not appear to be a special transport mechanism or system engaged in the transfer of orotic acid. It is most probably carried across the cell membrane by some of the systems engaged in the non-specific transport of anions. Orotic acid injected in tracer amounts does not test the capacity of the transporting mechanisms. As a result, orotic acid is immediately transformed in the cell to pyrimidine nucleotides. In this case the specific radioactivity of RNA will depend on the size of the immediate cellular pool after orotic acid transformation, and not on the uptake of orotic acid. Under tracer conditions this uptake is assumed to operate so efficiently that all the administered dose is immediately converted into pyrimidine nucleotides. In other words, orotic acid transport across the cell membrane and the rate of its incorporation into RNA pyrimidine nucleotides is not regulated by the rate at which it moves into the kidney cells. In the case of castrated animals the kidney contains almost the same amount of DNA (total per organ) as the larger kidney from the normal animal. If we now assume that every kidney cell takes up the same amount of orotic acid, then it is not difficult to visualize that there is much more radioactivity per gram of kidney tissue in castrated animals than in normal animals, simply because there are many more cells in the same volume. In this way, by administering the same amount of radioactive orotic acid to normal and castrated animals it is possible to create a false impression of the increased rate of pyrimidine synthesis in the

acid-soluble pool. Our present results do not show any difference in the rate of isotope disappearance from the acid-soluble pools of normal and castrated mice, whatever this might mean. Even if the rate of disappearance of radioactivity were higher in the kidneys of castrated mice it would not mean that there is a faster turnover of the acid-soluble fraction. It can be demonstrated, theoretically, that the same amount of radioactivity disappears faster from a smaller pool than from a larger one.

The situation in the mouse liver is even more complicated. Castration did not produce the same results here as in the mouse kidney. Whereas there is practically no difference in the specific radioactivity of the various RNA fractions between normal and castrated mice, there is much more radioactivity, when expressed per cell or per mg of DNA, in the livers from normal animals. (Of course, comparisons of DNA content and the number of cells in an organ such as the liver are subject to extensive criticism and discussion.) There is also a pronounced difference in the rate of disappearance of radioactivity from RNA fractions in the liver and kidney of the same animal. It is assumed that the liver is much more actively synthesizing proteins than the kidney. From this standpoint the rate of incorporation of radioactive orotic acid into the mouse kidney is even more difficult to understand, unless we assume different rates of reutilization of the precursor in these two organs. It is evident that estimation of turnover rates of RNA molecules from experiments *in vivo* requires a more carefully defined system with more precise knowledge of specific radioactivities and concentrations in every step of RNA precursor biosynthesis.

In the present work I have also described the results of fractionation of the liver and the kidney under the conditions in which the mice were deprived of the endogenous sources of androgen hormones. The relative proportions of free to bound ribosomes were preserved in almost every fraction, except for a slight difference in livers of castrated mice. To what extent this difference represents a real situation in the mouse liver after castration is difficult to determine.

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