THE INCORPORATION OF LABELED METHIONINE INTO PROTEIN BY PITUITARY TISSUE*

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In recent years numerous reports on the uptake of labeled amino acids by the proteins of tissue preparations have appeared. Various amino acids have been used, and the reaction has been shown to occur in tissue slices (1–4), homogenates (5), and in resting bacteria (6, 7). Considerable evidence has accumulated, indicating that this labeling of protein represents a synthesis of peptide bonds. In particular, it has been established that the labeling reaction, with apparently a single exception (8), depends on the simultaneous oxidation of a suitable metabolite, that the label cannot be removed by prolonged dialysis (9), and that, when carboxyl-labeled amino acids were used, the label is not removed by treatment with ninhydrin unless the protein is first subjected to hydrolysis (10). It seems clear that the labeling reaction can be used to measure the over-all activity of the enzymes involved in the synthesis of protein.

This report concerns the incorporation of $^{35}$S-labeled methionine into proteins, rat pituitary tissue being the source of the protein-synthesizing enzymes. It might be expected that this tissue, being specialized in the production of protein hormones, would be highly active in the incorporation of amino acids into protein, and this has been found to be the case. In addition, the effect of various physiological conditions which are known to be related to pituitary function has been investigated.

The use of methionine as the labeled substance in such studies has been investigated by Tarver and his coworkers (2, 9). These workers have stressed the error introduced by the conversion of the methionine to cystine and the subsequent labeling of protein by the formation of disulfide bonds (1). In this work this error was eliminated by removal of the mercaptans present in the proteins as the cuprous salts. Thus, only radioactivity incorporated as methionine was determined.

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† Portions of this work were taken from the thesis submitted by Michael Halikis in partial fulfilment of the requirement for the degree of Master of Science.
EXPERIMENTAL

Albino rats of the Sprague-Dawley strain were used throughout this work. The majority were raised in our colony and were fed standard fox chow pellets supplemented with leafy vegetables. A few of the rats were obtained from a local dealer and had been fed Rockland rat diet supplemented with soy bean meal prior to purchase. All rats were fed ad libitum up to the time of sacrifice. Except in the experiments in which the effect of age was investigated, all the animals were adults of over 150 gm. in weight and were in good health.

The labeled methionine used in this work was obtained from Dr. D. L. Tabern of the Abbott Laboratories. A paper chromatogram, supplied with the compound, indicated it to be free of other labeled sulfur compounds.

The animals were sacrificed by a blow at the back of the neck which killed them within 10 seconds in an attempt to minimize the stress reaction. The pituitary glands were removed and weighed on a torsion balance. Except for the very smallest glands (less than 1 mg.) they were all cut in half. They were then placed in a Warburg flask which contained Ringer's bicarbonate solution (11) supplemented with sodium succinate. The side arm contained the S\textsuperscript{35}-labeled methionine dissolved in Ringer's bicarbonate. The flasks were placed in a water bath maintained at 37.5° and shaken at 2 revolutions per second.

After filling the flasks with a mixture of 95 per cent oxygen-5 per cent carbon dioxide by evacuating twice with a vacuum pump, the methionine was tipped in to start the reaction. The final concentration of methionine was \(0.5 \times 10^{-3}\) M, and that of succinate was \(7.8 \times 10^{-3}\) M, in a total volume of 1 ml., the pH being 7.4. 10 minutes was the maximum length of time from the moment of sacrifice until the reaction was started. The amount of tissue varied from 5 to 15 mg. in wet weight.

The reaction was halted at the end of 2 hours by the addition of 1 volume of 0.67 M trichloroacetic acid (TCA). The contents of the flask were quantitatively transferred to glass homogenizers (12) and homogenized thoroughly. They were then transferred to centrifuge cones; 0.33 M TCA was used liberally to effect the transfers. The precipitated proteins were washed once with TCA at the centrifuge. The precipitates were then dissolved in 0.1 M NaOH and reprecipitated with TCA (final concentration was always 0.33 M). The solution was allowed to stand for 1 hour to insure complete separation of the proteins. This step was repeated once. After the final centrifugation, the proteins were hydrolyzed by the addition of 2 ml. of a solution containing 12 per cent formic acid in concentrated hydrochloric acid, which is recommended by Miller and du Vigneaud (13) and others (14) to minimize humin formation.
Hydrolysis was effected by heating under an air condenser for 15 hours at 100°. At the end of this period, the hydrolysates were evaporated to dryness under reduced pressure.

The labeled mercaptans produced under the conditions described were removed by the method of Zittle and O'Dell (15) which involves precipitation of the insoluble cuprous salts. Inactive cystine was added as a carrier prior to addition of the cuprous oxide. This treatment removes cysteine, cystine, and homocystine, leaving the methionine in the supernatant solutions.

The supernatant solutions were concentrated on a steam bath, and then transferred with the aid of slightly acidified water to planchets made of Parafilm, the bottoms of which were completely covered with Whatman No. 1 filter paper. Final evaporation of the planchets occurred at room temperature. The samples were counted with a proportional counter and an internal counting tube. A study of this method of preparing samples has recently been completed by Goldkamp and Melchior.

Control samples were run by precipitation of the tissue by the addition of TCA immediately after adding the labeled methionine. This type of control was employed by Simpson and Tarver (9) and was referred to as the "zero time control." The procedure described above effectively washed out all of the physically absorbed methionine, since essentially no activity was found in the zero time controls. If the precipitated proteins are not dissolved as described, or are dissolved only once, errors of up to 50 per cent can be introduced due to adsorption or occlusion of methionine by the protein precipitate.

The addition of succinate was found to cause an increase in protein labeling of approximately 40 per cent over that observed when glucose was used as the energy source. This was true of both liver and pituitary preparations. Hence, succinate was added routinely to all the flasks as described.

For convenience, we have defined the unit of activity of the enzymes under study as the number of micromoles of $^{35}$S incorporated as methionine (i.e., not precipitated by cuprous ion) in 2 hours under the conditions described. All values are expressed in these units in Tables I to IV so that they can be directly compared.

Results

As the pituitary is a tissue specialized in the synthesis of protein hormones, it was expected that it would be highly active in the enzymes under study. The activity per gm. of tissue was compared to liver slices prepared from the same animals. As can be seen from the results (Table I)

1 Manuscript in preparation.
the pituitary is indeed much more active in the incorporation of methi-
onine than liver. This is even more striking when calculated on the basis
of dry weight, since the pituitary averages about 16 per cent solids, com-
pared to 27 to 30 per cent for liver. The relative activities of various
tissues in this regard have been compared by Tarver (16). Referring to
these data, it is apparent that pituitary tissue is the most active of any
tissue reported so far in this respect. In addition, a few measurements
were made on the separated anterior and posterior lobes of the glands.
As can be seen from Table I, over 90 per cent of the activity is incorpo-
rated into the anterior lobe, which appears to be somewhat more active
per gm. than the posterior portion. However, the exact evaluation of
the data on the posterior lobe is in question because of its minute size.
In all the other experiments described here whole glands were used.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Average weight of gland*</th>
<th>Units† per gm. tissue</th>
<th>Average weight of gland*</th>
<th>Units† per gm. tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior pituitary</td>
<td>10.8</td>
<td>0.156</td>
<td>9.9</td>
<td>0.172</td>
</tr>
<tr>
<td>Posterior &quot;</td>
<td>1.3</td>
<td>0.085</td>
<td>1.1</td>
<td>0.069</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>0.049</td>
<td></td>
<td>0.049</td>
</tr>
</tbody>
</table>

* Two to three glands were pooled in the experiments on pituitary.
† A unit is defined as the micromoles of methionine incorporated into protein
in 2 hours under the conditions described in the text.

Effect of Age

In studying the effect of age on the activity of the pituitary, male rats
were used whose weight varied from 10 to 375 gm. The activities in
various groups have been averaged and are presented in Table II. The
activity of glands from very young animals was approximately 33 per
cent greater than that of the older animals. After the first 2 weeks,
however, the activity remains relatively constant throughout life, with
some tendency to decrease in the older rats.

If it can be shown that these measurements are related to the ability of
the gland to produce its protein hormones, it seems likely that the total
activity of the gland in a given animal is of greater significance than the
activity per unit weight of tissue. In Fig. 1 the activity in total units per
gm. of animal is plotted against the body weight. It can be seen that
the youngest animals show approximately 100 per cent greater activity
than any of the others, with some tendency for a decrease in activity in
the older rats.
TABLE II

Effect of Age

<table>
<thead>
<tr>
<th>Rat weight</th>
<th>No. of determinations</th>
<th>Units per gm. pituitary*</th>
</tr>
</thead>
<tbody>
<tr>
<td>gm.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10–15</td>
<td>3†</td>
<td>0.209 ± 0.022</td>
</tr>
<tr>
<td>20–50</td>
<td>4†</td>
<td>0.145 ± 0.008</td>
</tr>
<tr>
<td>100–160</td>
<td>4</td>
<td>0.156 ± 0.006</td>
</tr>
<tr>
<td>240–275</td>
<td>4</td>
<td>0.122 ± 0.011</td>
</tr>
<tr>
<td>300–375</td>
<td>4</td>
<td>0.134 ± 0.010</td>
</tr>
</tbody>
</table>

* Expressed as units ± the standard error of the mean.
† Two to six animals were used per determination.

Fig. 1. Uptake of methionine by pituitary of male rats. A unit is defined as the micromoles incorporated in 2 hours under the condition described in the text.

TABLE III

Effect of Sex and Estrous Cycle

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Units per gm. pituitary</th>
<th>Units per kilo rat X 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female (14)</td>
<td>0.128 ± 0.006</td>
<td>6.56 ± 0.426</td>
</tr>
<tr>
<td>Male (7)</td>
<td>0.144 ± 0.007</td>
<td>4.68 ± 0.303</td>
</tr>
<tr>
<td>Female, proestrus (7)</td>
<td>0.120 ± 0.011</td>
<td>5.96 ± 0.495</td>
</tr>
<tr>
<td>&quot; diestrous (7)</td>
<td>0.135 ± 0.007</td>
<td>7.17 ± 0.743</td>
</tr>
<tr>
<td>Multiparous (7)</td>
<td>0.129 ± 0.003</td>
<td>7.81 ± 0.526</td>
</tr>
</tbody>
</table>

* The number of separate determinations is given in parentheses.

Effect of Sex

The activity of the male and female glands is essentially the same per unit weight of tissue (see Table III). However, when the data are calcu-
lated in terms of total units per gm. of animal, the females have a gland that is about 30 per cent more active than that of the male, and this difference is highly significant. Calculation by the Fisher t method (17) indicates that there is less than one chance in a hundred that this is not a real difference (i.e., p is less than 0.01).

Fig. 2. Sex difference in the weight of pituitary glands from albino rats. Each point represents an average for a number of animals. The spread of both body and gland weights in the groups selected is indicated by drawing the lines one standard error on each side of the mean for the particular group.

Table IV

<table>
<thead>
<tr>
<th>Condition</th>
<th>Units per gm. pituitary</th>
<th>Units per kilo rat $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy (6)</td>
<td>0.148 ± 0.019</td>
<td>5.83 ± 0.867</td>
</tr>
<tr>
<td>Lactation (6)</td>
<td>0.101 ± 0.005</td>
<td>4.23 ± 0.158</td>
</tr>
<tr>
<td>Normal female (14)</td>
<td>0.128 ± 0.006</td>
<td>6.56 ± 0.426</td>
</tr>
</tbody>
</table>

* The number of separate determinations is given in parentheses.

Thus it is apparent that the pituitary of the female has more total activity than the male of similar size, although the activity per gm. of tissue is essentially identical. This is in accord with the available data on the relative size of the glands, shown in Fig. 2. The weights of the pituitary in males and females are similar up to a body weight of about 100 gm. After this, the female gland is generally heavier than that of the male. There was no apparent relationship to other variables such as estrus, pregnancy, or lactation. This is in essential agreement with the data of Hatai (18).
The various stages of the estrous cycle had no apparent effect on the activity of these enzymes. Measurements were made during diestrus and during proestrus, the stage being determined by vaginal smear as described by Long and Evans (19) (see Table III). In addition, several animals were studied that were known to have borne four to six litters during their life. It is interesting to note that they still exhibited a normal estrous cycle and that the glands of these animals show the same overall activity as those of younger animals.

Effect of Pregnancy and Lactation

The activity of the glands from normal and from pregnant rats falls within the same range, as shown in Table IV. In lactation, however, the picture is quite different. It was found that the lactating gland has a lower activity than any other group, and this difference is significant ($p = 0.02$). The same relationships hold when the data are expressed in terms of total units per kilo of animal.

DISCUSSION

On the basis of the work presented here, it is premature to attempt to relate the incorporation of a labeled amino acid in vitro to the function of the pituitary in the production of protein hormones. However, the relatively great activity of this tissue with respect to other tissues, and the differences observed under various physiological conditions related to pituitary activity, make it interesting to compare these findings with the activity of the gland as established by other techniques.

It is apparent from the foregoing that the pituitary gland in the rat shows an essentially constant activity of the enzymes involved in the incorporation of methionine into protein. The only conditions in which there is a significant difference in this variable are the first few days of the animal's life, when a higher activity is observed, and lactation, when there is a significant loss in activity.

During the initial stages of the animal's life, it would seem that the pituitary is primarily secreting the growth hormone. The growth rate of the animal is rapid throughout this period, and, if calculated in terms of percentage increase per day, diminishes rapidly from the time of birth. The growth rates of albino rats have been given by Donaldson (20) (see also King (21)). It is interesting to note that at about the 18th to the 21st day, when the activity tends to level off, the rat pituitary is known to undergo profound alterations. Jailer (22) has shown that it is at this point that the gland first begins to contain adrenocorticotropic hormone, and Swezy (23) has shown that the gland first begins to produce gonadotropic hormones at this age. The enzymes measured in this work appear
to diminish rapidly in activity during the initial stages of the animal's life until approximately this age, when they appear to level off and stay relatively constant throughout life.

The effect of the estrous cycle on the pituitary of the rat has been difficult to assess, because of the very short cycle and lack of knowledge of the time required for the gonadotropic hormones to act. Histological studies have indicated a difference between male and female glands; however, the male gland has a relatively higher percentage of α- and β- cells, with corresponding fewer chromophobes (24). These differences are not reflected in the ability of the tissue to incorporate methionine into protein.

The state of activity of the pituitary during lactation has been studied by Hurst and Turner (25). They report a surge of lactogenic hormone production during the first 3 days after parturition. The content of lactogenic hormone then diminishes slowly. The measurements reported here were made 7 days after parturition. The observed decrease in activity is perhaps related to the fact that the estrous cycle is halted during this period (19). It is evident that the lactating female exhibits a total activity per gm. of animal similar to that found in the normal male, both being significantly lower than that of the normal female. The lower total activity in the male results from the smaller size of the pituitary, whereas that in the lactating female results from a less active gland.

The results obtained during pregnancy were more variable than those of other groups. This is possibly explained by the fact that these animals were in all stages of pregnancy, from 3 to 21 days. One would expect major changes to be occurring in the pituitary during this period. It seems possible that a more thorough study of pregnancy would show significant differences between the various stages. It should be noted that no correction for the weight or the possible pituitary activity of the litter was taken into consideration in these experiments.

Further interpretation of the significance of these results awaits determination of which proteins become labeled. Work is in progress to fractionate the pituitary tissue after the labeling has occurred.

SUMMARY

1. Measurements have been made of the ability of pituitary tissue to incorporate a labeled amino acid into protein in vitro. The method described permits measurements with single rat pituitaries.

2. Pituitary tissue was found to be much more active in the incorporation of the label than liver.

3. The activity was significantly greater in young animals. After the
first 2 weeks of life the activity remained relatively constant, with some
tendency to decrease in the older rats.

4. No significant differences were found during the phases of the estrous
cycle or during pregnancy.

5. The male gland had the same activity per gm. as the normal adult
female; because of its smaller size, the total activity in the male was sig-
nificantly less than that in the female.

6. During lactation a significant decrease in the ability of the gland
to label protein was observed.

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