CCXLIX. THE RAT UNIT OF TESTICULAR HORMONE.

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IN previous papers [Korenchevsky, 1932, 1, 2; Korenchevsky, Dennison and Schalit, 1932] a detailed description has been given of the method of assay of testicular hormone, using rats as the experimental animals, and a new rat unit was suggested for use in the assay of this hormone.

In the present investigation a test was made of the method of assay and the suitability of this rat unit, and a comparison of the size of the rat unit (RU) with that of the capon unit (CU).

Technique.

Experiments were performed on 42 rats belonging to 8 litters. The technique of the assay, described previously, was followed strictly. The ages of the rats at castration, the number of days after castration before the injections were started, and the weights of the rats at the end of the experiments are given in Table I.

Table I. *Age and average final weights of the rats in each group of each litter.*

No. of litter	No. of rats in litter	Age at castra- tion (days)	No. of days after castration injections started	Weight of rats (g.)					
				Control rats	Rats injected with				
					1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu
1	6	24	50	221	230	234	—	—	—
2	7	24	50	197	182	204	—	—	—
3	5	27	43	166	184	182	—	—	—
4	3	29	32	174	—	—	193	—	—
5	6	27	32	217	—	—	—	218	210
6	5	27	32	194	—	—	209	—	—
7	5	22	32	192	—	—	—	191	—
8	5	25	75	343	—	—	—	387	—

A well-purified testicular hormone, isolated from urine, belonging to one and the same batch, prepared by Messrs Schering Ltd., was used for the injections¹. The hormone was dissolved in oil and its strength was stated to

¹ We wish to express our thanks to Messrs Schering Ltd. for kindly supplying this preparation.

be equal to 25 capon units per cc., as standardised in the Schering Laboratories. We have not re-tested the preparation on capons, and in the following its strength will be designated in capon units according to Schering's standardisation.

For the injection of small doses, sufficient of the original preparation for the day's injection was diluted, each morning, with sesame oil under aseptic precautions. For the injection of 5.0 and 7.5 cu daily the undiluted original preparation was used. The daily dose was injected subcutaneously twice a day, in half doses, at about 10 a.m. and 5 p.m. for 7 consecutive days.

After the morning injection the remaining doses for the evening injection were covered with sterilised glass lids and placed in the cold store. In no case were indurations or any local reactions noticed, nor was there depression of the animals or of their appetite.

All the litters were castrated before sexual maturity at the age of 22 to 29 days.

As can be seen from Tables II to VI, only the first 7 litters were used in the standard test. These were chosen intentionally so that the number of days after castration was between 32 and 50 days and therefore the weights of the rats did not differ greatly—the average weights at the end of the experiment being between 166 and 234 g.

Litter 8, which was about double the weight of the other litters (see Table I), was chosen to show the correlation between the dose and the weight of the rat. The results of the injections into the rats of this litter are discussed below in a separate paragraph and are not included in Tables II to VI in order to avoid confusion. The supply of testicular preparation was unfortunately insufficient for the treatment of more than one litter of large rats.

The influence on prostate with seminal vesicles.

The results obtained are summarised in Table II. In previous work [Korenchevsky *et al.*, 1932] experiments showed that the variations in the increase of prostate with seminal vesicles were not large in rats belonging to different litters injected at a similar period after castration with the same dose of the same testicular extract, provided the rats were operated on at the same time in relation to sexual maturity. The same results were obtained in the present experiments, as can be seen from a comparison of the percentage increase of prostate with seminal vesicles of rats injected with the same dose but belonging to different litters (Table II). The variation was considerable in only one group of the rats injected with one cu. However, it must be remembered that this, the smallest dose of one unit, would give an increase comparatively close to the limit of physiological variations [Korenchevsky, 1932, 2] of the weight of prostate with seminal vesicles. In a standardisation using the smallest dose, therefore, it is sometimes difficult in a biological test to rely on an increase of 20 or 30 mg. in the weight of the organs, since physiological variation of a few mg. will influence the results obtained considerably.

Table II. *Influence on prostate with seminal vesicles.*

The average weights, actual and calculated, per 200 g. of body weight, of the prostate and seminal vesicles of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Con- trol rats	Rats injected with					Percentage increase in weight				
		1 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu	1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu
A. Actual weights.											
1	64	97	105	—	—	—	51.5	64.0	—	—	—
2	62	81	96	—	—	—	30.6	54.8	—	—	—
3	71	100	117	—	—	—	40.8	64.8	—	—	—
4	56	—	—	120	—	—	—	—	114.1	—	—
5	55	—	—	—	160	219	—	—	—	190.8	298.0
6	56	—	—	111	—	—	—	—	98.2	—	—
7	53	—	—	—	154	—	—	—	—	190.6	—
Av.	60	93	106	116	157	219	41.0	61.2	106.2	190.7	298.0
B. Weight calculated per 200 g. body weight.											
1	58	85	90	—	—	—	46.5	55.2	—	—	—
2	63	90	94	—	—	—	42.8	49.2	—	—	—
3	87	109	129	—	—	—	25.3	48.3	—	—	—
4	64	—	—	124	—	—	—	—	93.8	—	—
5	51	—	—	—	146	212	—	—	—	186.2	315.5
6	58	—	—	106	—	—	—	—	82.9	—	—
7	55	—	—	—	162	—	—	—	—	194.5	—
Av.	62	95	104	115	154	212	38.2	50.9	88.4	190.4	315.5

To correct this at least 3 litters should be used and the averages can then be considered reliable and, as can be seen from the tables, this procedure gives satisfactory results. It would be still better to use for the standardisation doses of about 2 or 3 units when small physiological variations would be relatively less important.

In the previous experiments a close correlation of direct proportion was obtained between the increase in the dose of the hormone injected and the average increase of the prostate with seminal vesicles.

It can be seen from Tables II and V that the same correlation was found in the present experiments. At the same time, when the weights of the rats are so nearly the same as was the case in our experiments, the average percentage changes for the actual weights are very similar to those calculated for the same unit of rat's weight. In both our previous and our present experiments the unit of 200 g. of body weight was chosen, since this is the average weight of the rats most often used for the experiments.

The influence on the penis.

As was stated in the previous paper, it was found that the penis responds to the injection of testicular hormone more slowly and in a less regular way than the prostate with seminal vesicles. However, as is shown in Tables III and V, the present experiment corroborates our previous statement, that, for the assay, the changes in the penis give an extra proof of the activity and strength of the preparation tested.

Table III. *Influence on the penis.*

The average weights in mg., actual and calculated per 200 g. of body weight, of the penis of the rats in each group of each litter and their percentage increase after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage increase in weight				
		1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu	1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu
A. Actual weights.											
1	82	97	98	—	—	—	18.3	19.5	—	—	—
2	67	79	80	—	—	—	17.9	19.4	—	—	—
3	67	80	103	—	—	—	19.4	53.7	—	—	—
4	67	—	—	104	—	—	—	—	55.2	—	—
5	64	—	—	—	110	137	—	—	—	71.9	114.0
6	70	—	—	111	—	—	—	—	58.6	—	—
7	66	—	—	—	110	—	—	—	—	66.6	—
Av.	69	85	94	108	110	137	18.5	30.9	56.9	69.3	114.0
B. Weights calculated per 200 g. body weight.											
1	74	85	84	—	—	—	14.8	13.5	—	—	—
2	68	87	78	—	—	—	27.9	14.7	—	—	—
3	82	87	113	—	—	—	6.1	37.8	—	—	—
4	77	—	—	108	—	—	—	—	40.3	—	—
5	59	—	—	—	100	131	—	—	—	69.5	122.0
6	73	—	—	100	—	—	—	—	37.0	—	—
7	69	—	—	—	115	—	—	—	—	66.7	—
Av.	72	86	92	104	108	131	16.3	22.0	38.7	68.1	122.0

Table IV. *Influence on the thymus.*

The average weights of the thymus (mg.) calculated per 200 g. body weight of the rats in each group of each litter and their percentage decrease after the injection of testicular hormone.

No. of litter	Control rats	Rats injected with					Percentage decrease in weight				
		1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu	1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu
1	508	398	410	—	—	—	21.6	19.3	—	—	—
2	644	508	529	—	—	—	21.1	17.9	—	—	—
3	569	564	525	—	—	—	0.9	7.7	—	—	—
4	639	—	—	611	—	—	—	—	4.4	—	—
5	850	—	—	—	616	607	—	—	—	27.5	28.6
6	702	—	—	666	—	—	—	—	5.1	—	—
7	862	—	—	—	734	—	—	—	—	14.8	—
Av.	682	490	488	639	675	607	14.5	15.0	4.8	21.2	28.6

In the most recent paper [Korenchevsky, 1932, 2] it was stated that the weighing of the dissected penis is more accurate if it is fixed in Bouin's fixative before weighing (*i.e.* the penis should be treated in the same way as the other organs). In this way more regular changes after injection were obtained in the penis.

From Tables III and V it is clear that, although the response of the penis to the injections was less sensitive, this organ, like the prostate with seminal vesicles, showed on the average changes in weight in direct proportion to the dose injected; for each RU or CU an increase was obtained in the weight of the

Table V. *The relation between the rat unit and the capon unit of testicular hormone.*

Dose of cu injected	Average % increase in weight		% increase obtained per cu		No. of rat units in dose
	Obtained	Expected	With each dose	Average (max. % deviations in brackets)	
Prostate + seminal vesicles:			A. Actual weights.		
1.0	41.0	—	41.0	40.4 (+ 5.2) (- 5.7)	1.03
1.5	61.2	61.5	40.8		1.53
2.5	106.2	102.5	42.5		2.66
5.0	190.7	205.0	38.1		4.77
7.5	298.0	307.5	39.7		7.45
Penis:					
1.0	18.5	—	18.5	18.2 (+ 25.3) (- 23.6)	—
1.5	30.9	27.8	20.6		—
2.5	56.9	46.3	22.8		—
5.0	69.3	92.5	13.9		—
7.5	114.0	138.8	15.2		—
B. Weights calculated per 200 g. body weight.					
Prostate + seminal vesicles:					
1.0	38.2	—	38.2	37.5 (+ 12.3) (- 9.6)	0.96
1.5	50.9	57.3	33.9		1.27
2.5	88.4	95.5	35.4		2.21
5.0	190.4	191.0	38.1		4.76
7.5	315.5	286.5	42.1		7.89
Penis:					
1.0	16.3	—	16.3	15.3 (+ 6.5) (- 11.1)	—
1.5	22.0	24.5	14.7		—
2.5	38.7	40.8	15.5		—
5.0	68.1	81.5	13.6		—
7.5	122.0	122.3	16.3		—

penis of about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

We would, therefore, advise the use of the changes in the penis as a rough extra proof of the activity and strength of the hormone. In the case of the penis the weights calculated per 200 g. of body weight gave closer results than the changes in the actual weights (Table V).

Influence on the thymus.

The changes observed in the thymus after the injection of testicular hormone are given in Table IV. Since these changes were small, to economise space only the weights calculated per 200 g. of rat's weight are given.

The decrease in the thymus after the injection of the extract used was in most cases small, and in one case (litter 3, dose 1 cu) even negligible; also the correlation of direct proportion between the increase in the dose injected and the degree of decrease is not clearly shown. In the previous experiments similar changes were noted. Nevertheless, in spite of the unsuitability of these changes for standardising the hormone, we would suggest that they be noted during an assay of a preparation as indicating the activity of that preparation on the thymus.

The influence on fat deposition, adrenals, thyroids and hypophysis.

As was found in previous experiments, seven days of injection is too short a period for the production of marked changes in these organs and in fat deposition. For this reason only the average figures of the results obtained with each dose are given (Table VI).

Table VI. *Average percentage changes in weight (actual and per 200 g. of rat's weight) of retroperitoneal fat, adrenals, thyroid and hypophysis of the injected rats compared with those of the control rats.*

Organs	Rats injected with				
	1.0 cu	1.5 cu	2.5 cu	5.0 cu	7.5 cu
Retroperitoneal fat:					
Actual weight	+ 37.4	+ 37.0	+ 13.3	- 14.6	- 15.9
Per 200 g. rats weight	+ 34.2	+ 39.0	+ 6.5	- 14.5	- 12.4
Adrenals:					
Actual weight	- 1.1	+ 2.1	- 8.2	- 9.4	- 16.7
Per 200 g. rats weight	- 4.3	- 5.0	- 16.0	- 10.0	- 13.2
Thyroid:					
Actual weight	+ 7.6	+ 7.6	+ 6.3	+ 9.0	+ 10.9
Per 200 g. rats weight	+ 4.1	+ 0.3	- 1.5	+ 8.5	+ 13.4
Hypophysis:					
Actual weight	- 1.2	- 5.2	- 9.4	+ 2.8	- 8.5
Per 200 g. rats weight	- 2.6	- 11.2	- 19.4	+ 1.1	- 6.8

It has already been stated [Korenchevsky, 1932, 1, p. 420] that, in order to be certain that the changes found after injection are outside the limits of physiological fluctuations, the increase in weight of the thyroids should be not less than 40 %, while for the decrease in weight of the adrenals, hypophysis and fat deposition an even larger figure should be allowed. The changes obtained in the present experiment are therefore too small to permit of any definite conclusions. We would like to note, however, that, though small, there was, in most cases, an increase in the weights of the thyroids and a decrease in the weights of the adrenals and hypophysis, that is, there was a tendency for the organs changed by castration to return to normal.

One more point must be mentioned here, that in castrated rats a period of 1 or 2 months after castration is not sufficient to produce maximum changes in the above organs [Korenchevsky, 1931, 2]. It is therefore necessary, in testing the influence of testicular hormone on these organs, to choose rats which have been castrated not less than 3 months, preferably more, before the test. For the same reasons the changes in fat deposition were also small, while the physiological variations in fat deposition were greater than in any of the organs which have been mentioned [Korenchevsky, 1932, 1]. However, it is noteworthy that, with small doses (1.0 or 1.5 cu) fat deposition increased and with large doses (5 or 7.5 cu) decreased. The intermediate dose of 2.5 cu produced a small increase in fat deposition. If these results are not accidental, they may indicate that metabolism is differently influenced by small and by large doses of testicular hormone.

The rat unit and its relation to the capon unit.

It must be emphasised that, since we did not re-test the capon unit of the preparation used, this unit was taken to be as was stated by Messrs Schering. Up to the present time the method of assay of testicular hormone by using capons has not been standardised and therefore the capon unit may vary in different laboratories.

With reference to the disadvantages of the capon unit, which have already been discussed [Korenchevsky, 1932, 1, p. 413], it is necessary to add that Womack and Koch [1932, p. 275] have established that the urine of pregnant and of normal women yields "the same amount of comb growth-stimulating material as urine from normal mature men." Kabak [1931] arrived at a similar conclusion. Womack and Koch (p. 276) state that "we have present in urine from pregnant and normal mature women, a substance very similar to or identical with the testicular hormone¹ but that it is different chemically in some respects and acts differently physiologically from the female sex hormone which produces oestrus in the castrated female. The actual source and function of this hormone is at present a mystery." It is important that the authors failed to obtain this hormone from the urine of an ovariectomised woman, which would indicate that the ovaries are the source of this hormone. The specificity of the comb test, therefore, becomes doubtful in several instances and in any case it seems that for the assay of testicular hormone it should be used simultaneously with, if not replaced by, a test on the sexual and other organs of castrated mammals. Taking this into consideration it is necessary to discuss the results of our present experiments with reference to the suitability of the method of assay and of the rat unit as these have been described in our previous and present papers.

In order to answer this question, the results are presented in Table V so as to show the relation between the capon and rat units and the suitability of the latter. It is clear, from the second and third columns of this table, that the increase in weight of the prostate with seminal vesicles obtained in the experiment is very close to that theoretically expected, since there is a direct relationship between the increase in the amount of hormone injected and the increase in the weights of the prostate with seminal vesicles. The fourth and fifth columns show that the percentage deviations from the average increase, corresponding to 1 CU, were not more than 6 % for the actual figures or 12 % when calculated per 200 g. of rat's weight.

This may be considered quite satisfactory for a biological test. The figures in the sixth column of the table were obtained by dividing "% increase obtained" (2nd column) by 40, since the suggestion for the rat unit was the increase in weight of the prostate with seminal vesicles by 40 % as compared with those of the uninjected control rats. As can be seen from the table, the rat units were nearly equal to the capon units. They are more nearly so if we

¹ As far as can be understood from the paper the only test made was that of comb growth in capons.

take the average figure: thus the sum of the doses in CU (column 1) is 17.50 CU, with which corresponds (column 6) 17.44 RU obtained by changes in the actual weight of the prostate with seminal vesicles, or 17.09 RU, obtained by the changes per 200 g. of body weight. From this, in the batch tested, 1 CU corresponds to 0.997 RU (actual weights) or 0.977 RU (per 200 g. of body weight).

Therefore, using our method of assay and definition of the rat unit, this latter proved unexpectedly to be nearly equal to the capon unit (as assayed in the Schering Laboratory).

Precautions necessary in assaying the rat unit.

1. All the precautions and rules detailed in the previous papers are confirmed.

2. *The influence of the difference in weight of the rats.* Since the rats must be castrated before the 30th day after birth, and since the weight of the prostate with seminal vesicles remains constant (per unit of body weight) from the end of the first month after castration [Korenchevsky, 1932, 2], the rats seem most suitable for the assay at the age of about 2 months. A longer period is inconvenient since it means waiting longer until the animals are ready for the assay. In our present experiments we used, without appreciable change in the results, rats castrated 32 to 50 days before the experiments were started. This margin of about 18 days is convenient since even in large Institutions, having their own breeding rooms, it is difficult to obtain at short notice the necessary number of litters of a special age.

The amount of the testicular hormone preparation available was unfortunately only sufficient for the performance of one experiment (on litter No. 8) for studying the influence of the same dose of hormone on rats weighing about twice as much as litters 5 and 7. The weights of the rats of these litters and their ages are given in Table I. The daily dose injected was 5 CU. The results on litter 8 are given in Table VII and, as can be clearly seen, the increase in weight of the prostate with seminal vesicles was considerably less than that in litters 5 and 7 injected at the same time with the same dose, but more than in litters 4 and 6 injected with half the dose (2.5 CU).

This indicates that:

(1) if the same dose of the hormone is injected into rats of different weights, the influence will be less in heavier rats;

(2) a strict direct proportion does not exist between the weight of the rat and the effect of the dose injected per unit of body weight, for in this case the increase of prostate with seminal vesicles instead of 154 % (actual weight) and 123 % (per 200 g. body weight) would be equal to that of the rats injected with 2.5 CU, *i.e.* about 106 % and 88 % respectively, since the weight of litter 8 was about twice that of litters 4 and 6.

Since, however, the experiment was performed on only one litter more experiments must be made before drawing a definite conclusion.

Table VII. *Influence of injections of 5 CU per day on average weight of organs of rats of litter 8 as compared with that of non-injected litter-mates.*

	Prostate +seminal vesicles		Penis		Thymus		Retroperitoneal fat	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual g.	Per 200 g. body weight g.
Control rats	87	51	88	51	480	278	17.92	10.51
Injected rats	221	114	157	81	484	250	18.28	9.46
% change in weight of organs	+154	+123	+78.4	+58.8	+0.8	-10.1	+2.0	-10.0

	Adrenals		Thyroid		Hypophysis	
	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.	Actual mg.	Per 200 g. body weight mg.
Control rats	66.3	38.5	28.1	16.4	11.5	6.7
Injected rats	63.8	32.9	35.8	18.6	14.7	7.6
% change in weight of organs	-3.8	-14.5	+27.4	+13.4	+27.8	+13.4

3. The dissection of the very atrophied prostate and seminal vesicles from the surrounding fat tissue is a rather delicate task and needs some training. It must be performed with the utmost accuracy, using, if necessary, a dissecting lens, and since these organs in castrated animals weigh only from 50 to 70 mg. the error in leaving a little fat tissue or removing a little of the prostatic tissue might influence the results considerably. At the same time accuracy must not interfere with rapid dissection as the organs must not be allowed to dry in the air, thus creating another source of error.

The accurate removal of fat tissue from all organs is roughly indicated by their sinking at once to the bottom of the vessel containing the Bouin's fixative. If any organ floats it must be removed from the fixative and the fat tissue left unnoticed removed.

For dissecting the hypophysis a very sharp small scalpel should be used.

4. The hormone is usually dissolved in oil and during injection it is necessary to push the needle into the subcutaneous tissue as far as possible from the point of entrance into the skin by pressing the bulb of the needle forward against the skin. In rats, if this is not done, about 15 to 30 minutes after the injection the oil begins to leak out and the loss in this way might be considerable. As a routine our rats are inspected about 45 minutes after the injections and any leakage is noted in the records.

5. The experience of Institutes breeding their own stocks of rats has shown that even with the best care it is not possible to prevent the occurrence of single cases of pneumonia among the rats. Rats suffering from pneumonia, especially those failing to grow or losing weight, must be discarded or the

results obtained with these rats must be taken with reservation, since pneumonia interferes with the influence of the injection of testicular extract. At *post mortem*, therefore, the lungs of the rats must always be examined for pneumonia.

Disadvantages of the method.

For each assay new animals must be operated on and chosen with reference to their age at castration and at the beginning of the experiment.

Advantages of the method.

1. The method of assay is accurate, precise in end-point reading, quick and simple, since it only requires the weighing of the organs and not a microscopic examination.

2. The data obtained give a direct indication of the changes in the secondary sexual organs of the male, *i.e.* the method is specific for the male hormone.

3. The method, when used for longer periods, gives an indication of the changes in most of the other organs and functions which are influenced by castration and usually, even after only 7 days, those in the thymus.

4. In order to determine the number of rat units contained in the dose injected at least 3 litters, preferably more, are injected with the same amount of any reasonable dose of the active material. Since the correlation between the increase of the dose of the injected hormone and the average degree of increase of prostate with seminal vesicles is one of direct proportion, the quotient of division of the average increase of the prostate and seminal vesicles by 40 will be the number of rat units injected (see Table V). In our experiments a test, from this point of view, was made only of doses from 1.0 to 7.5 RU. Whether the direct proportional correlation will remain true for much higher doses has yet to be investigated.

SUMMARY.

1. Using 42 castrated rats belonging to 8 litters a test was made of the method of assay of testicular hormone and the suitability of the rat unit, as defined in previous papers, and a comparison was made between the rat unit and the capon unit.

2. From the results obtained, both the method used and the suggested rat unit seem to be satisfactory for the assay of testicular hormone.

3. The rat unit suggested is the minimum daily dose, which, when injected twice a day in half doses during 7 consecutive days, into at least 3 litters of rats castrated before the 30th day after birth, will produce on the average an increase of 40 % in the weight of the prostate with seminal vesicles as compared with the average weight of these organs in the uninjected litter-mates. For the assay it seems most convenient to use rats about 30 to 50 days after castration.

4. The rat unit proved to be nearly equal to the capon unit (as assayed in the Schering Laboratories).

5. On the average, a correlation of direct proportion was found between the increase of the dose, from 1 to 7.5 RU, and the degree of average increase in the weight of prostate with seminal vesicles and, less sensitively, of the penis.

6. For each RU or CU the increase in weight of the penis was about 18 % (actual weight) or 15 % (when calculated per 200 g. of body weight) as compared with that of the controls.

7. The response of the thymus to the injections of the hormone was variable and less sensitive.

8. Although, for assaying testicular hormone, a rat unit is suggested which is based on the more sensitive response of the prostate with seminal vesicles, it is also useful to note the changes in weight of the penis and thymus as supplementary proofs of the activity of the preparation.

9. The precautions which are necessary for obtaining accurate results in the assay are detailed, with special reference to the weight and age of the rats used for assay.

10. The disadvantages and advantages of the proposed method of assay and of the rat unit are discussed.

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