XLIII. THE RESPONSE OF RATS TO GRADED DOSES OF FLAVIN

By HANS LINDHOLM

From the College of the Pharmaceutical Society, London

(Received 18 December 1937)

IN view of the desirability of adopting a standard of reference for determining the flavin content of food substances, an experiment was carried out to see whether a graded response to graded doses of flavin could be obtained, i.e. to see whether a curve of response to doses of flavin could be constructed. Increase in weight of rats was chosen as the criterion for the measurement of the response. In a preliminary experiment we fully confirmed the findings of other workers that a supplement of 10 γ flavin given to rats fed on a diet containing abundance of vitamin B₁ but very little or none of the other B-vitamins, did not produce any increase in weight, though it prevented loss of weight and death [Kuhn *et al.* 1933, 1, 2, 3, 4; Kuhn & Wagner-Jauregg, 1933; Chick *et al.* 1935; Györgyi, 1935; Halliday & Evans, 1937]. To produce an increase in weight another factor is required by rats. Halliday & Evans [1937] have shown this factor to be present in fresh liver and in an aqueous liver extract. The writer found that this "liver factor" when given alone as an aqueous extract after treatment with fuller's earth, in doses corresponding to 2 g. fresh liver, did not produce any increase in weight in the rats. When, however, both flavin and liver extract were given to the rats, they increased in weight rapidly. In the main experiment, therefore, all the rats were given a daily dose of liver extract corresponding to 2 g. fresh liver. It was then found that the response of the rats was graded to the dose of flavin given. Curves of response for male and female rats respectively relating increase in weight in 3 weeks to dose were logarithmic in shape and of slopes very similar to those constructed by Coward [1933] relating increase in weight in 3 weeks to the dose of vitamin A given.

EXPERIMENTAL

Young rats weighing about 60 g. were used. Since it is essential, in constructing a curve of response to doses of any vitamin, to supply in liberal amounts all other substances necessary for the well-being of the animal, the basal diet used in this experiment consisted of:

Caseinogen (physiological caseinate, Glaxo)	15 %
Dextrinized rice starch	77 %
Agar-agar	2 %
Salt mixture (Steenbock's 40)	4 %
Vitamin B ₁ powder (B.D.H. 100 I.U. per g., i.e. about 12 I.U. per rat per day) ...	2 %

In addition each rat was given 6 drops of a good sample of cod liver oil twice a week.

Four rats were housed in a cage about 12 in. square and 6 in. high during the preparatory period. Each rat was housed in a separate cage 9 × 12 × 6 in.

during the time that doses were given. All cages were provided with grids of wire mesh standing about 1 in. from the floor.

The rats were weighed after 1 week of feeding on the basal diet, then twice a week and more frequently when they were becoming steady in weight. During the time that doses were given in the preliminary experiment, the rats were weighed daily, but in Exps. 1 and 2 they were weighed twice a week. On an average the rats ceased to grow after about 30 days, the males reaching an average weight of 82 g. and the females 75 g. They were considered ready for experiment when they had been steady in weight, or declining slightly, for more than 8 days.

Preliminary experiment

A preliminary experiment was carried out to determine (a) whether the basal diet chosen was suitable for this work, (b) how much of the B factors other than vitamin B₁ and flavin were necessary in order to obtain the maximum response to the graded doses of flavin to be given in the main experiment, i.e. to obtain the steepest possible curve of response. Four groups of 6 rats each were prepared by feeding them on the basal diet until they became steady in weight. The different groups were then given:

- Group 1. No supplement.
- „ 2. 10γ flavin daily.
- „ 3. 1 ml. of a liver extract daily (≡ 2 g. fresh liver).
- „ 4. 10γ flavin + 1 ml. liver extract daily.

Flavin. A solution of lactoflavin kindly supplied by the A/S Ferrosan, Copenhagen, was used. It was said to contain about 500γ lactoflavin per ml. (determined photochemically as lumiflavin), a little vitamin B₁ and that part of “vitamin B₆” which is adsorbed with flavin on the fuller’s earth used in its preparation from whey. The solution had been sterilized by filtration, and to it had been added 0.2% tricresol. It was stored in brown sealed ampoules in the dark. 0.02 ml. of this solution containing 10γ was given directly into each rat’s mouth from an Agla syringe fitted with a blunt injection needle.

Liver extract. The liver extract was prepared from hog’s liver. The liver was minced, extracted with cold water containing 2 ml. *N* H₂SO₄ per l. (4 l. to 1.2 kg. liver), heated almost to boiling-point and, after cooling, was filtered on a Büchner funnel and washed. This was repeated three times; the combined extracts were concentrated *in vacuo* to 1 l., and the pH was adjusted to 4.0–4.5 with *N* H₂SO₄. This extract was intimately mixed with 250 g. fuller’s earth specially prepared for adsorption purposes, and was allowed to stand at 0° until next day, during which time it was stirred frequently. The fuller’s earth was then filtered off on a Büchner funnel and washed with water; the filtrate was again treated with 250 g. fuller’s earth with stirring for 24 hr. as before. It was again filtered and washed. The filtrate was concentrated *in vacuo* to such a volume that 1 ml. solution corresponded to 2 g. fresh liver. Four such preparations were made during the course of this investigation. The dose given to each rat was considered to be quite sufficient and there was no evidence in these experiments that any one of the extracts was less potent than the others.

In Fig. 1. are drawn the composite weight curves of the 4 groups of rats. It may be seen that:

(a) the basal diet is satisfactory since the rats, after the first 4 days, lost weight steadily; one of the six rats in this group died after 8 days;

(b) the dose of flavin chosen, 10γ, maintained the weight of the rats for 21 days;

(c) the dose of liver extract, 1 ml., equivalent to 2 g. fresh liver, maintained the weight of the rats for 21 days;

(d) the rats which were given doses of both flavin and liver extract grew rapidly, 28 g. in 21 days.

Fig. 1. Average increase in weight of rats given basal diet: (1) with no supplement, (2) with a supplement of 10 γ flavin, (3) with a supplement of 1 ml. liver extract ($\equiv$ 2 g. fresh liver), (4) with a supplement of 10 γ flavin + 1 ml. liver extract.

The fact that neither flavin alone nor the liver extract alone produced any increase in weight, while the two combined did produce an increase in weight, confirms the findings of the workers mentioned at the beginning of this paper. It indicated that the preparations available (basal diet, liver extract and flavin solutions), were suitable for the attempt to construct a curve of response to graded doses of flavin.

Experiment 1

Forty rats were prepared as in the preliminary experiment. They were then divided into 6 groups and given graded doses of flavin, every rat of any one group being given the same dose.

Group 1. No dose.

„ 2. 1.0 γ flavin.

„ 3. 2.5 γ flavin.

Group 4. 5.0 γ flavin.

„ 5. 10.0 γ flavin.

„ 6. 20.0 γ flavin.

Every rat of each group received 1 ml. of the liver extract daily.

The average increases in weight of the male and female rats respectively in each group were calculated. The results are summarized in Table I. It may be seen that the response is graded to the dose of flavin given. Moreover, when the responses are plotted against the logarithms of the doses of flavin given, the curves for both male and female rats are straight lines (Fig. 2). The equations representing the best straight lines drawn between the points relating increase in weight and the logarithms of the doses of flavin given were found to be:

$$y = 2.95 + 24.04x \quad (\text{male rats}),$$

$$y = 5.53 + 15.85x \quad (\text{female rats}).$$

Table I. *Average increase in weight in 3 weeks of groups of rats given graded doses of flavin*

Sex	No. of rats	Dose of flavin γ	Av. increase in wt. in 3 weeks g.
♂	5*	0	-7.3
	5†	1.0	6.25
	5	2.5	6.5
	5	5.0	19.8
	5	10.0	27.8
	5	20.0	36.2
♀	2	0	-8.5
	3†	1.0	9.0
	3	2.5	5.0
	3†	5.0	17.5
	3	10.0	23.0
	3	20.0	27.0

* Two rats of this group died within 14 days.

† One rat from each of these groups died before the end of the experiment and has not been included in the average increase in weight.

Fig. 2. Curves of response of (a) male, and (b) female rats relating mean increase in weight in 3 weeks and the logarithm of the dose of flavin given daily. $\times$ Males. $\oplus$ Females.

Therefore the equations representing the curves relating increase in weight in 3 weeks and doses of flavin given are:

$$y = 2.95 + 24.04 \log x \quad (\text{male rats}),$$

$$y = 5.53 + 15.85 \log x \quad (\text{female rats}).$$

It is interesting to note that the slopes of these curves are very similar to the slopes of the curves relating increase in weight in 3 weeks and doses of vitamin A given, found by Coward [1933].

The curves of response may be used for determining the flavin content of a substance, just as the curves for other vitamins are used. One dose of the standard and only one dose of the substance under examination need be tested, the relative values of the doses being calculated from the abscissae of the curves (for male and female rats) corresponding to the increases in weight produced by the doses.

Experiment 2

One determination of the flavin content of a food substance, fresh unpasteurized Jersey milk, was made by this "increase in weight" method. Four groups of rats were prepared as above and given the following daily doses, every rat of any one group being given the same dose.

Group 1.	7.5 γ flavin.	Group 3.	5 ml. milk.
„ 2.	30.0 γ flavin.	„ 4.	10 ml. milk.

This experiment was begun before it was known what the response to 20 γ flavin might be. The doses of milk were chosen from the statements in the literature that milk contains about 1 γ flavin per ml. It was hoped that the responses to the 2 doses of milk would lie on the curves (for male and female rats) obtained from the doses of flavin given in this experiment. The results are summarized in Table II.

Table II. *Average increases in weight in 3 weeks of 2 groups of rats given graded doses of flavin and of 2 groups given graded doses of Jersey milk*

	Sex	No. of rats	Dose	Av. increase in wt. (g.)
Flavin	♂	5	7.5 γ	21.6
		5	30.0 γ	38.4
	♀	1	7.5 γ	14.0
		3	30.0 γ	23.0
Milk	♂	3	5.0 ml.	23.6
		3	10.0 ml.	48.0
	♀	4	5.0 ml.	37.7
		3	10.0 ml.	35.3

The curves of response relating increase in weight in 3 weeks and doses of flavin were found in this experiment to be:

$$y = -2.8 + 27.9 \log x \quad (\text{male rats}),$$

$$y = +0.9 + 14.9 \log x \quad (\text{female rats}).$$

It is evident from the coefficients of x that these curves have very nearly the same slopes as the curves in the first experiment. They are not in exactly the same positions, however, which is good evidence that there are fluctuations in the responses of animals at different times to the same doses of flavin, even when the preparation of the animals is as nearly standardized as possible. This emphasizes the need for a simultaneous test on a standard preparation of flavin whenever the flavin content of a substance is to be determined biologically. It is impossible to determine the flavin content of a substance by an animal response without a simultaneous test of a standard.

The flavin content of the milk was determined by the following calculation. Since only a small number of rats was available for the test, a combined curve of response for the male and female rats which were given doses of 7.5 and 30 γ flavin was constructed. The responses of the male and female rats given each

dose of flavin were averaged without taking into consideration the number of male or female rats in each group. Thus the final average response to each dose was calculated as if from equal numbers of male and female rats. The curve was found to have the following shape: $y = -0.9 + 21.4 \log x$. The results from the 2 doses of milk were treated similarly. The average response of the rats given 5 ml. milk at the same time that the doses of flavin were being tested was 30.6 g., that of the rats given 10 ml. was 41.6 g. The abscissa of the combined curve corresponding to an increase in weight of 30.6 g. was 29.6; therefore 5 ml. of milk contained 29.6 γ flavin and 1 ml. apparently contained 5.9 γ flavin.

Similarly the abscissa corresponding to an increase of 41.6 g. was 96.8; therefore 10 ml. of milk contained 96.8 γ flavin, and 1 ml. apparently contained 9.7 γ flavin. Considering the small number of rats used for each dose of milk (7 for the dose of 5 ml. and 6 for the dose of 10 ml.), these results may be considered to be in fairly good agreement, and the average value may be expressed as about 7.8 γ flavin per ml.

The accuracy of the method

The standard deviation of a single observation was determined from all groups of animals that had received 1 ml. liver extract per day. There were 45 male rats in 10 groups containing more than 1 rat per group and 22 female rats in 8 groups containing more than 1 rat per group. σ for the male rats was found to be 7.65 and for the female rats 5.44. The probable error of determinations made with 2 groups of 10 rats each, one group being used for the standard and the other for the test substance, was calculated from the standard deviation and the slope of the curve of response. It was found that with these numbers, if male rats were used for the test, the probable error was +24.2 or -19.5 %. With female rats, the probable error was +26.5 or -20.9 %. This degree of accuracy compares favourably with that of the 3 weeks' increase in weight method for determining vitamin A as used in this laboratory.

Dermatitis

The rats given no dose or only a small dose of flavin in this experiment (but given the liver extract) developed dermatitis to a degree equal to that designated stage I or II by Halliday & Evans [1937]. Their fur became greasy, their forelegs, ears and sometimes the whole head became blood-stained. These signs were sometimes apparent when the rats were considered ready for the test, i.e. when they had become steady in weight; sometimes the signs were manifest only towards the end of the dosing period. The larger doses (10, 20 or 30 γ) of flavin cured or prevented this condition. It is, however, uncertain whether this was due to the flavin or to some impurity in the flavin solution. Milk also cured or prevented the development of dermatitis. Loss of hair on the backs and forelegs accompanied the development of dermatitis and was also cured by the larger doses of flavin solution. Post-mortem examination (macroscopic) of all the rats revealed no specific lesions. The rats which had received no flavin, or only small doses, looked thin and ill-nourished.

SUMMARY

A preliminary experiment indicated that, with the basal diet used, a large daily dose of flavin (10 γ) or a large daily dose of liver extract (equivalent to 2 g. fresh liver) prevented loss of weight and death of young rats for 3 weeks

after they had ceased to put on weight on the basal diet alone. Other rats given doses of both flavin and liver extract not only lost no weight but grew rapidly.

A curve of response was constructed relating increase in weight to dose of flavin given, all the rats being given daily doses of liver extract equivalent to 2 g. of fresh liver. Groups of 7 or 8 rats were given daily doses of 0, 1.0, 2.5, 5.0, 10.0 or 20.0 γ flavin, and the average increases in weight of male and female rats respectively were calculated and plotted against the logarithms of the doses of flavin given.

The curves of response were found to be represented by the equations

$$\begin{aligned}y &= 2.95 + 24.04 \log x & (\text{male rats}), \\y &= 5.53 + 15.85 \log x & (\text{female rats}).\end{aligned}$$

The standard deviations of the responses of male and female rats were found to be 7.65 and 5.44 respectively. The probable error of the test (1 chance in 2) was found to be +24.2 or -19.5 % for the male rats and +26.5 or -20.9 % for the female rats.

One sample of milk (fresh unpasteurized Jersey) was found by this "increase in weight" method to contain 7.8 γ flavin per ml.

Dermatitis of a mild degree developed in the rats given no dose, or only a small dose, of flavin; it was cured or prevented by the larger doses of flavin preparation or by milk.

REFERENCES

- Chick, Copping & Edgar (1935). *Biochem. J.* **29**, 722.
 Coward (1933). *Biochem. J.* **27**, 445.
 Györgyi (1935). *Biochem. J.* **29**, 741.
 Halliday & Evans (1937). *J. biol. Chem.* **118**, 255.
 Kuhn & Wagner-Jauregg (1933). *Ber. dtsh. chem. Ges.* **66**, 1577.
 — Györgyi & Wagner-Jauregg (1933, 1). *Ber. dtsh. chem. Ges.* **66**, 317.
 — — (1933, 2). *Ber. dtsh. chem. Ges.* **66**, 576.
 — — (1933, 3). *Ber. dtsh. chem. Ges.* **66**, 1034.
 — Rudy & Wagner-Jauregg (1933, 4). *Ber. dtsh. chem. Ges.* **66**, 1950.