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# Effect of Aza-Substituted Nucleotides on the Starve–Refeed Response of Rats (38595)

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In nutritional experiments with animals, substances used for the purpose of demonstrating a requirement for *de novo* protein synthesis must meet the following two criteria: (a) A demonstrable interference with protein synthesis at some level, and (b) relatively small or no effect on food intake. Most of the substances used in bacterial work to inhibit protein synthesis cannot be used in animal experiments, because of a failure to meet either criterion a, b, or both. One of the antibiotics which has been used in bacteria as well as in rats is 8-azaguanine. This substance is believed to be similar enough to guanine to be incorporated into RNA, but the resultant RNA appears to be nonfunctional (1–3). It was found that 8-azaguanine affects food intake only to a comparatively small extent (4); its effect of inhibiting the enzyme overshoot in the liver of starved–refed rats was not duplicated by pair-feeding (4). The inhibition of the enzyme overshoot by 8-azaguanine is considered to signify a dependence on *de novo* RNA synthesis (4).

In the studies reported here the suitability of other aza-substituted nucleotides in animal experiments was investigated. The starve–refeed regimen was used as in previous studies (4) since the enzyme overshoot in the liver can be produced after 2 days of refeeding: A period not excessively long but requiring that the rats maintain adequate food intake during the 48 hr of refeeding. Within the context of these experiments a substance will be referred to as "effective" or "suitable" for nutritional experiments if it were as effective as 8-azaguanine in inhibiting the enzyme overshoot and caused no greater decrease in food intake than did 8-azaguanine. A substance will be referred to as "unsuitable" or "toxic" if it caused a greater reduction in food intake than did 8-azaguanine.

*Materials and Methods. Animals and animal care.* Specific pathogen-free, male Wistar rats were purchased from either Manor Research Company[1] of New Brunswick, NJ, or from Hilltop Laboratory Animals, Inc. of Scottdale, PA. The rats were shipped in such a manner as to prevent stress due to heat, infection, lack of food or water. The animals from Manor Research Company were used in experiments summarized in Table I, while the animals purchased from Hilltop Laboratory Animals, Inc. were used in experiments summarized in Table II.

Rats were housed individually in screen-bottom stainless-steel cages. Environmental conditions were rigidly controlled by automatic devices regulating temperature, humidity and the light–dark cycle. Lights were off between 6 PM and 6 AM.

The experimental protocol was as follows: One group was fed *ad libitum* the "inducer diet" (65G, or high-glucose diet) containing 65% glucose, 25% casein, 5% corn oil, 4% Jones–Foster Salt Mix, and 1% Vitamin Diet Fortification Mixture. Diet ingredients were purchased from Nutritional Biochemicals Co. of Cleveland, OH. The remaining groups were starved 2 days and refed the 65G diet for 2 days.

*Drug treatment.* The analogs were administered during both days of the refeeding. 8-Azaguanine, 8-azaadenine, 8-azahypoxanthine, and 8-azaxanthine were dissolved in dilute alkali (NaOH, pH = 10), 2-azauridine, 6-azauridine, 5-azacytidine, and 5-azauracil were dissolved in distilled water. 8-Azaguanine, 8-azaadenine, 8-azahypoxanthine, 8-azaxanthine, 2-azauridine, and 6-azauridine were purchased from K&K Laboratories, Inc. of Plainview, NY. 5-Azacytidine was purchased from Terra-Marine

[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

TABLE I. EFFECT OF ANTIBIOTICS ON THE STARVE-REFEED RESPONSE.

| Treatment | RLS[a] | G6PD[a] units | ME[a] units | Total liver fat | IRI[a] units |
|---|---|---|---|---|---|
| | | per 100 g body wt | | mg per 100 g body wt | per ml serum |
| Experiment 1 | | | | | |
| *Ad libitum*-fed | 5.31 ± 0.25[b] | 22.4 ± 2.0 | 14.5 ± 1.4 | 181 ± 10 | 33 ± 3 |
| Starved 48 hr | 3.41 ± 0.02 | 6.3 ± 2.5[c] | 5.2 ± 0.5[c] | 126 ± 10[c] | 21 ± 4[c] |
| Refed 48 hr | 5.84 ± 0.10 | 57.6 ± 2.5[d] | 30.0 ± 1.4[d] | 507 ± 10[d] | 26 ± 3 |
| 8AG | 5.97 ± 0.20 | 23.4 ± 3.4 | 13.7 ± 3.4 | 185 ± 15 | 28 ± 4 |
| 2AU | 5.77 ± 0.10 | 65.5 ± 3.1[d] | 25.9 ± 1.3[d] | 342 ± 45[d] | 29 ± 5 |

[a] Abbreviations used: RLS = relative liver size = (liver wt × 100)/body wt; G6PD = glucose 6-phosphate dehydrogenase; ME = malic dehydrogenase (NADP-linked); IRI = serum immunoreactive insulin; 8AG = 8-azaguanine; 2AU = 2-azauridine.

[b] Standard error of mean.

[c] Values smaller than in *ad libitum*-fed animals ($P < 0.05$).

[d] Values larger than in *ad libitum*-fed animals ($P < 0.05$).

TABLE II. EFFECT OF AZA-SUBSTITUTED NUCLEOTIDES ON THE ENZYME OVERSHOOT.[a]

| Nucleotide given during refeeding[c] | G6PD[b] units/100 g body wt | ME[b] |
|---|---|---|
| Experiment 2 | | |
| None | 91.8 ± 5.6[d] | 39.4 ± 2.6 |
| 8-Azaguanine | 35.3 ± 6.5[e] | 22.4 ± 3.0[e] |
| 8-Azahypoxanthine | 93.7 ± 4.3 | 39.4 ± 3.0 |
| 8-Azaxanthine | 106 ± 11 | 46.5 ± 5.5 |
| Experiment 3 | | |
| None | 89.4 ± 9.8 | 46.7 ± 5.8 |
| 6-Azauridine | 41.3 ± 3.8[e] | 28.3 ± 2.7[e] |
| 5-Azauracil | 78.7 ± 3.6 | 44.0 ± 2.4 |

[a] Rats were starved 2 days and refed 2 days the 65% glucose diet.

[b] Abbreviations: G6PD = glucose 6-phosphate dehydrogenase; ME = malic enzyme.

[c] The substituted nucleotides were injected intraperitonally, 0.5 ml between 8–10 AM and 0.5 ml between 8–10 PM during both days of refeeding. One ml solution contained 15 mg of nucleotide. The substituted purines were dissolved in dilute alkali (pH = 10, NaOH), while the substituted pyrimidines were dissolved in water. It was found that the effect of the alkali was negligible on food intake and enzyme responses.

[d] Standard error of mean. Numbers represent the mean of five animals/group.

[e] Differs significantly from group given no nucleotide ($P < 0.05$).

Bioresearch of La Jolla, CA, while 5-azauracil was purchased from Sigma Chemical Co. of St. Louis, MO. The analogs were administered intraperitoneally in two equal daily doses (7.5 mg/dose) between 8–10 AM and 8–10 PM. The rationale for selecting these dose levels was as follows: In previous studies it was shown that the effect of 8-azaguanine was approximately the same in the dose range of 7.5–30 mg/rat/day (4). By selecting an intermediate dose (one commonly employed in the author's laboratory) the small variation in dose/g rat due to variation in body size would be negligible. Also, it was felt that doses significantly smaller than 7.5 mg/rat/day might be insufficient to cause the maximal inhibitory effect and thus render the question of suitability difficult to answer. Similarly, very large doses would have to be considered impractical because of the potentially deleterious effect of the large amount of alkali necessary to dissolve such nucleotides as 8-azaguanine and 8-azaadenine.

Rats were killed by one of two methods. In the first experiment, rats were anesthetized with an intraperitoneal injection of 90 mg of sodium amytal/kg body wt. The thoracic cavity was opened after the blink reflex could no longer be elicited and a 2–4 ml blood sample was collected by heart puncture. Blood samples were centrifuged at 2° for 30 min at 3000 rpm; the serum was collected and frozen until used in the determination of serum immunoreactive insulin (5). The liver was excised, chilled and weighed. In experiments 2 and 3, rats were stunned by a sharp blow to the head and killed by decapitation. The carcasses were

then exsanguinated, and the liver was removed, chilled and weighed.

*Enzyme assay.* Glucose 6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49) and malic enzyme (ME) (EC 1.1.1.40) activities were assayed as previously described (6). We found that livers could be frozen up to 3 mo without appreciable loss in G6PD and ME activity. Some of the livers (i.e., experiments 2 and 3) were, therefore, kept frozen until the enzyme assays were performed.

*Units and calculations.* Enzyme activity is expressed as units/100 g body wt. The justification for the use of this method of reporting enzyme activity has been previously presented (7). Enzyme activity can be recalculated as units/g liver by dividing the mean enzyme activity tabulated in the tables by the corresponding value of RLS. One unit of enzyme is defined as that amount of enzyme which can produce 1 μmole measured product per minute under the conditions of the assay. Differences between the treatment groups were tested by Student's *t* test. Body weight changes (as percent/day) and food intake (as g food eaten/100 g body wt/day) were calculated but are not tabulated.

*Remarks on some aspects of the procedure.* It was determined in separate experiments that injections of small amounts of alkali (0.5 ml, NaOH, pH = 10 at 8 AM and 8 PM for 2 days) or 90 mg sodium amytal/kg body wt (before killing) did not alter enzyme activity.

*Results.* Administration of 8-azaguanine and 2-azauridine affected the enzyme response in the starved–refed rats as follows (Table I): the enzyme overshoot was prevented by 8-azaguanine, but not by 2-azauridine. Serum immunoreactive insulin (IRI) was decreased by starvation and returned nearly to normal in refed rats at the time of the measurement. 8-Azaguanine and 2-azauridine had no noticeable effect on serum IRI. Unexpectedly, both 8-azaadenine and 5-azacytidine proved to be highly toxic, resulting in complete aphagia and death within 12 hr. Reducing the dosage of 8-azaadenine and 5-azacytidine to 1.5 mg/rat/day or even to 500 μg/rat/day improved survival but rats still ate very little food. It was concluded, therefore, that these antibiotics are unsuitable for studies where the animal needs to eat.

The cause of 8-azaadenine toxicity was then investigated. There were two possible alternatives to be considered: (a) That toxicity was due to 8-azaadenine itself or, (b) that toxicity was due to a breakdown product. In the breakdown of adenine the first product is hypoxathine which is then converted to xanthine before further metabolism (8). The first breakdown product of guanine is xanthine (8). It was expected that the breakdown of the aza-substituted adenine and guanine would follow a similar pathway. Therefore, if 8-azaadenine toxicity were due to alternative 2, 8-azahypoxanthine should be very toxic, while 8-azaxanthine should be no more toxic than 8-azaguanine, since every metabolite following and including 8-azaxanthine is common to both 8-azaadenine and 8-azaguanine breakdown. On the other hand, if 8-azaadenine toxicity were due to 8-azaadenine itself then neither 8-azahypoxanthine nor 8-azaxanthine should be toxic. To differentiate between these two possibilities, experiment 2 was performed (Table II). In this experiment the effects of 8-azaguanine, 8-azahypoxanthine and 8-azaxanthine are compared. 8-Azaguanine was effective in preventing the enzyme overshoot, but 8-azahypoxanthine and 8-azaxanthine had no measurable effect on either the enzyme overshoot or the food intake. These results indicate that the toxicity of 8-azaadenine is due to the effects of 8-azaadenine itself.

The cause of 5-azacytidine toxicity was similarly investigated. First, we wished to ascertain if toxicity was associated with substitution at the fifth position Attempts to convert 5-azacytidine to 5-azauridine by sodium nitrite were not successful. In a further attempt at elucidating the cause of 5-azacytidine toxicity, starved–refed rats were injected with 6-azauridine or 5-azauracil. The results of these experiments (experiment 3) are summarized in Table II. Again, there could be two alternate explanations of 5-azacytidine toxicity: (a) That toxicity is due to 5-azacytidine itself or (b) that toxicity is due to a breakdown product. Since 5-azauracil would be expected as a product along the degradation of 5-azacytidine and since 5-azauracil was both ineffec-

tive in preventing the enzyme overshoot and nontoxic, we may conclude that the toxicity of 5-azacytidine is due to 5-azacytidine itself. Interestingly (and unexpectedly) the overshoot was prevented by 6-azauridine. In rats treated with 6-azauridine, food intake was decreased by only 10–12%. It appears, therefore, that 6-azauridine may also be useful in animal studies.

*Discussion.* The toxicity of 8-azaadenine can be readily explained by the extremely wide involvement of adenosine triphosphate and cyclic AMP in virtually every aspect of intracellular and intercellular regulation and life processes. An adequate explanation for 5-azacytidine toxicity, on the other hand, is somewhat more difficult. Cytidine is a known constituent of a certain class of lipids involved with the nervous system (8). Interference with vital nerve functions could account for the cause of 5-azacytidine toxicity. But there is no evidence at present as to whether 5-azacytidine is incorporated into lipids.

5-Azacytidine has been used in enzyme induction experiments (9–11). Notably, the activities of tyrosine aminotransferase (9, 10) and uridine kinase (11) are elevated by treatment with 5-azacytidine. There is some question as to the mechanism of action of 5-azacytidine in these cases. It is possible that 5-azacytidine is incorporated into RNA resulting in unreadable RNA as obtained after 8-azaguanine administration (10). Such RNA may be involved in the formation of a translational repressor as proposed in the regulation of tyrosine aminotransferase (12). Under these conditions the uridine kinase mRNA would be stabilized. This mechanism is supported by the fact that the affect of 5-azacytidine is inhibited by actinomycin D (11) and cytidine (11) indicating that the effect of 5-azacytidine is dependent on the phosphorylation and incorporation of the nucleotide into RNA made *de novo*. The effect of cycloheximide in increasing uridine kinase activity with or without 5-azacytidine (11) could be due to inhibition of enzyme breakdown. However, the lack of effect by 8-azaguanine on uridine kinase (11) is not possible to reconcile with this explanation at the present time.

It appears then that of the five substituted nucleotides (8-azaadenine, 5-azacytidine, 8-azaguanine, 2-azauridine, and 6-azauridine) only 8-azaguanine and 6-azauridine are suitable for nutritional experiments. 5-Azacytidine is suitable only for experiments in which food intake is not required.

*Summary.* Rat liver glucose 6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) activities were increased by starvation–refeeding to levels above those found in rats fed *ad libitum*. The increases in enzyme activities above *ad libitum*-fed levels were prevented by 8-azaguanine and 6-azauridine, but not by 2-azauridine. Blood insulin levels were not affected at the time studied. Two aza analogs, 8-azaadenine and 5-azacytidine, proved to be too toxic in this type of studies. Since 8-azahypoxanthine, 8-azaxanthine and 5-azauracil were neither effective in preventing the enzyme overshoot, nor toxic to the animals, it was concluded that the toxicity to the animals of 8-azaadenine and 5-azacytidine is due to the compounds themselves rather than to their breakdown products.

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