various types of cancers, surveillance of AS and DB usage in hospital populations should be continued.

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References and Notes

1. Wisconsin Alumni Research Foundation, *Long Term Saccharin Feeding in Rats, Final Report* (WARF, Madison, Wis., 1973); Division of Pathology, Food and Drug Administration, *Subacute and Chronic Toxicity and Carcinogenicity of Various Dose Levels of Sodium Saccharin, Final Report* (Government Printing Office, Washington, D.C., 1973), pp. 169–170; D. L. Arnold, C. A. Moodie, N. C. Grice, S. M. Charbonneau, B. Stavric, B. T. Collins, P. F. McGuire, I. C. Munro, *Long Term Toxicity of Orthotoluenesulfonamide and Sodium Saccharin in the Rat: An Interim Report* (Toxicology Research Division, Health Protection Branch, National Health and Welfare Ministry, Ottawa, Canada, 1977).
2. S. Mondal, D. W. Brankow, C. Heidelberger, *Science* **201**, 1141 (1978).
3. R. M. Hicks, J. Chowaniec, J. St. J. Wakefield, in *Mechanisms of Tumour Promotion and Cocarcinogenesis*, T. Slaga, Ed. (Raven, New York, 1978), vol. 2, p. 475; S. M. Cohen, M. Arai, J. B. Jacobs, G. M. Friedell, *Cancer Res.* **39**, 1207 (1979).
4. D. Simon, S. Yen, P. Cole, *J. Natl. Cancer Inst.* **54**, 587 (1975); R. W. Morgan and M. G. Jain, *Can. Med. Assoc. J.* **111**, 1067 (1974); I. I. Kessler, *J. Urol.* **115**, 143 (1976); ——— and J. P. Clark, *J. Am. Med. Assoc.* **240**, 349 (1978).
5. G. R. Howe, J. D. Burch, A. B. Miller, B. Morrison, P. Gordon, L. Weldon, L. W. Chambers, G. Fodor, G. M. Winsor, *Lancet* **1977-II**, 578 (1977); A. B. Miller and G. R. Howe, *ibid.*, p. 1221.
6. E. L. Wynder and R. Goldsmith, *Cancer* **40**, 1246 (1977).
7. E. L. Wynder and S. D. Stellman, *Cancer Res.* **37**, 4608 (1977).
8. ——— *J. Natl. Cancer Inst.* **62**, 471 (1979).
9. Nonneoplastic tobacco-related diseases eligible for interview are myocardial infarction, abdominal aortic aneurysm, peripheral vascular disease, chronic obstructive pulmonary disease, chronic bronchitis, emphysema, gastric ulcer, and cirrhosis of the liver. In addition, many other nonneoplastic diseases are eligible as control diagnoses, including fractures, burns, infections, cataracts, surgical procedures, and so forth.
10. This apparent selection bias for males is likely to be due in part to the relative difficulty of treatment of bladder cancer, and the tendency of patients in higher socioeconomic strata to be referred to specialized hospitals, such as Memorial Hospital in New York, for primary care.
11. Proper interpretation of the data requires judgment as to whether bias in selection of control patients has been minimized and confounding reduced. The extent to which confounding factors are associated with the exposure of interest and also differ among cases and controls may profoundly influence estimates of association between exposure and disease. Both AS and DB use were strongly associated with socioeconomic status indicators such as occupation level (27 percent of professionals used AS compared to 12 percent of unskilled workers) and higher education (26 percent of college graduates used AS compared to 16 percent of those without high school). AS use was also greater among ex-smokers than smokers, owing partly to the higher degree of education among ex-smokers (and possibly to their fear of weight gain), but was low among heavy consumers of alcohol, users of nonfilter cigarettes, and male ward patients; among female ward patients AS use was higher, owing possibly to the greater level of obesity in this group.
12. Regular use was defined as continued use for at least one month. The reported frequency of "regular" AS or DB use lasting less than one year was < 1 percent.
13. Questions were also asked about use of cyclamates, which, in combination with saccharin, were contained in nearly all artificial sweeteners between 1960 and 1969. Saccharin has been the only AS permitted by federal law since the banning of cyclamates as a carcinogen in 1969. The number of subjects specifically recalling cyclamate use, as distinct from saccharin, was too small for analysis. However, since cyclamates would have been used by nearly all who used AS for 10 years or more, and since no increased bladder cancer risk was observed among this group, it is highly unlikely that cyclamate consumption contributed to excess cancer risk in our study population.
14. O. S. Miettinen, *Biometrics* **60**, 75 (1970).
15. Exclusion of patients with diabetes from the analysis resulted in negligible lowering of *RR*'s. Exclusion of patients with cardiovascular diseases also resulted in trivial changes; patients with cardiovascular disease in our study did not, as has often been suggested, consume larger amounts of AS or DB than other groups of controls.

    These findings are essentially in agreement with results obtained during an earlier phase of the study, in which a less elaborate questionnaire was used. In that version, three questions (history of regular AS use [yes or no], quantity, duration) were added in 1974 to a bladder cancer study already in progress, in response to widespread public interest in possible health effects of saccharin. That study, published in 1977 (*6*), found no association between AS and bladder cancer based on the rather limited numbers then available: 132 male and 31 female cases and equal numbers of matched controls. As data collection proceeded, an interim, preliminary analysis was submitted in a progress report to the National Cancer Institute in October 1977, based on 260 male and 86 female patients with bladder cancer. It gave crude *RR* estimates of 1.87 for males and 0.9 for females. Reservations were expressed at the time that a causal hypothesis might not be supported by the data for several reasons: (i) Substantial socioeconomic differences existed between cases and controls, with 29 percent of all male controls coming from Veterans Administration and county hospitals (notably Los Angeles County) compared with only 8 percent of male cases; opposite patterns of socioeconomic confounding appeared to explain much of the opposite female *RR* estimates. (ii) For a given substance to produce opposite risks for men and women would be highly unusual. (iii) *RR* estimates varied substantially from one city to another.

    When data collection terminated in 1977, bladder cancer cases numbered 402 males and 122 females, with 7200 male and 3777 female controls. Final crude *RR* estimates (and 95 percent confidence intervals) were 1.85 (1.45–2.36) for males and 0.99 (0.61–1.59) for females. When adjusted for age, hospital, hospital-room status, and year of interview, these *RR*'s fell to 1.43 (1.10–1.86) and 0.89 (0.48–1.64), respectively. A matched-pair analysis, with matching based on the above variables plus education, further reduced the *RR* estimates to the statistically not significant values of 1.13 (0.60–2.09) and 0.80 (0.20–2.98). An equivalent result was obtained by matching on occupation instead of education. There were no noteworthy or statistically significant differences between cases and controls with respect either to quantity or duration of AS use.
16. N. Mantel and W. Haenszel, *J. Natl. Cancer Inst.* **22**, 719 (1959).
17. All cigarette smokers interviewed had smoked for at least 10 years.
18. S. D. Walter, *Am. J. Epidemiol.* **105**, 387 (1977). The tables in this reference refer strictly to unmatched studies; for matched pairs of cases and controls, the least significant risks are slightly smaller.
19. I. I. Kessler, *J. Natl. Cancer Inst.* **44**, 673 (1970); B. Armstrong and R. Doll, *Br. J. Prev. Soc. Med.* **28**, 233 (1974).
20. An additional observation of a strong association between obesity and kidney cancer in both men and women leads to the prediction that these patients use substantial amounts of AS. Use of AS was in fact higher in the current series of 65 male kidney cancer patients, but not among the 88 male kidney cancer patients interviewed in 1974–1977. This epidemiological lead merits further investigation.
21. Supported by contract NO1-CP-55666 and grant CA-17613 from the National Cancer Institute and by grant RD-48 from the American Cancer Society. Computations were performed in part at the Department of Energy Mathematics and Computing Laboratory, Courant Institute for Mathematical Sciences, supported by DOE contract EY76-C-02-3077 at New York University, New York. The late Jerome Cornfield provided valuable counsel throughout this investigation. We thank E. Cuyler Hammond for critically reviewing the manuscript.

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Detrital Nonprotein Amino Acids Are the Key to Rapid Growth of *Tilapia* in Lake Valencia, Venezuela

Abstract. *The detritivorous cichlid* Sarotherodon mossambicus *grows rapidly on a low-protein diet by assimilating detrital nonprotein amino acids. Differences in the quantity of detrital amino acids in different lakes may account for the variable success of* S. mossambicus *introductions around the world.*

The world's most productive fish are the tilapias (family Cichlidae), which feed as detritivores and herbivores at the base of the aquatic food chain in tropical and subtropical freshwaters (*1*). The detritivorous species *Sarotherodon mossambicus* (= *Tilapia mossambica*) is of special interest. Hoping to benefit from its high productivity, man has extended the distribution of this species from a small endemic zone in southern Africa to many parts of the tropics and subtropics. The results have been mixed: some introductions established populations of rapidly growing individuals that reach a large size (> 2 kg); others resulted in populations of slowly growing, stunted individuals of no commercial value.

In an effort to explain this, the feeding ecology of *S. mossambicus* has been studied in detail (*2*). Two recent findings are of special significance for detritivorous fish in general. First, the diet of rapidly growing *S. mossambicus* in Lake Valencia, Venezuela, contains very little protein (*3*). Growth rate in animals is directly proportional to the amount of protein in the diet; judging from the nutritional requirements of other animals, protein levels in the diet of *S. mossambicus* should be adequate for maintenance only (*4*). How do these fish grow so well on a low-protein diet? Second, although the exceptionally long gut of detritivorous fish is assumed to be necessary for complete digestion, in *S. mossambicus* complete digestion and assimilation appear to occur in the first half of the intestine (*4*).

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Table 1. Amino acid composition of two detrital aggregate samples and of pooled stomach and posterior intestinal contents of *S. mossambicus* (N = 18) from Lake Valencia. Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; His, histidine; Lys, lysine; and Arg, arginine.

Sample	Milligrams per 100 milligrams of total amino acids															
	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
Detrital aggregate [grid 1, sample 5 (*3*)]	14.4	5.8	6.1	15.4	5.3	7.6	9.3	4.2	1.7	3.1	7.4	3.6	2.9	1.4	5.5	6.3
Detrital aggregate [grid 2, sample 5 (*3*)]	14.5	5.8	6.3	16.3	5.9	7.5	8.5	4.2	1.3	3.2	6.9	2.6	2.4	2.0	5.2	7.4
Stomach contents	11.7	6.0	5.5	14.5	4.1	6.6	7.9	5.9	1.6	4.7	8.6	3.3	2.4	2.4	6.5	8.4
Posterior intestinal contents	12.3	6.7	5.9	14.7	5.5	7.2	7.5	5.7	0	4.5	7.9	3.9	3.5	2.4	6.1	6.2

What is the function of the second half? Both questions are answered when the nutritional significance of nonprotein detrital amino acids is examined. In addition, the variable abundance of these amino acids in different lakes may explain the variable growth of *S. mossambicus* populations.

The results I report here primarily concern the rapidly growing, introduced population in Lake Valencia. Some comparison is made to the unusually slowly growing endemic population in Lake Sibaya, South Africa. In both lakes, *S. mossambicus* feeds on detrital aggregate—a mixture of algae, bacteria, and other detritus on the lake bottom and on the surfaces of aquatic plants (*4, 5*). Microorganisms comprise < 10 percent of the organic weight of this material; protein values are < 10 percent of the organic weight and account for less than one third of the organic nitrogen present. The form of the remaining nitrogenous organic matter is not known. Investigators studying similar material from other systems suggest that these compounds include polypeptides and nonprotein amino acids (*6*) and other nonprotein compounds such as amino sugars, phenol proteins, and nitrogen-containing humic acids (*7*). These compounds may be bound to organic or inorganic material. To establish the role of nonprotein amino acids in the nutrition of *S. mossambicus*, I have determined the amino acid composition of detrital aggregate, the quantity of nonprotein amino acids, and the extent to which these are assimilated by the detritivore.

Samples of food, gut contents, and feces used in previous assimilation studies (*4, 5*) and of detrital aggregate collected directly from the lakes (*3, 5*) were analyzed for total amino acid content. Amino acids were freed by acid hydrolysis under nitrogen (24 hours) and quantified by a fluorimetric technique calibrated with glycine (*8*). In this procedure, amino acids differ in the level of fluorescence they produce, so glycine fluorescence values were weighted by using the average amino acid composition of four samples as determined with an automated amino acid analyzer (*9*) and molar fluorescence equivalents for specific amino acids (*8*). Protein was quantified as described by Kauskik and Hynes (*10*), and amounts of nonprotein amino acid were calculated by subtracting protein amino acids from total amino acids. Assimilation efficiency (amount assimilated as a percentage of amount ingested) was measured with a modification of Conover's unassimilated ash ratio technique (*3, 4*).

Table 2. Quantities of protein and nonprotein amino acids assimilated by *S. mossambicus* in Lake Valencia.

Amino acid source	Milligrams per gram of				Assimilation efficiency (%)
	Amino acids in food ash	Amino acids in feces ash	Ash in food	Amino acids assimilated from food	
Protein	197	54	300	42	72.2
Nonprotein	674	246	300	128	63.5

The amino acid composition of detrital aggregate in Lake Valencia meets the nutritional requirements of fish (Table 1) (*11*). The composition varied little among the four samples and was similar to that reported for a variety of algae (*12*). Nonprotein amino acids averaged 14.6 percent of organic weight (range, 6.9 to 23.6 percent; N = 30)—approximately six times the amount of protein present (range, 0.5 to 5.1 percent; mean, 2.57 percent; N = 45). Assimilation efficiencies were 72.2 percent for protein and 63.5 percent for nonprotein amino acids (Table 2). Although assimilation of protein is more efficient, nonprotein amino acids contribute 76 percent of all amino acids assimilated by *S. mossambicus*. Thus I calculate that *S. mossambicus* in Lake Valencia assimilates 25.6 mg of amino acid for each kilojoule assimilated (*13*)—a level very similar to that required for maximum growth rates of trout and catfish under aquaculture conditions (*10*). Clearly, nonprotein detrital amino acids are the key to the rapid growth of *S. mossambicus* in Lake Valencia.

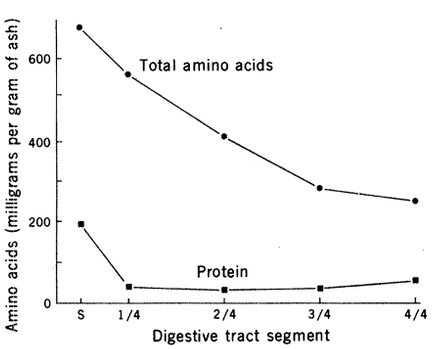

Fig. 1. Digestion and assimilation of protein and total amino acids from detrital aggregate by *S. mossambicus* in Lake Valencia. Samples are the contents of the stomach (*S*) and the four quarters of the intestine pooled from 18 fish.

This fish uses its extremely long intestine to assimilate the nonprotein amino acids (Fig. 1). Although protein digestion is completed in the first quarter of the intestine, nonprotein amino acids (totaling nearly three times the amount of digested protein) are gradually assimilated as the food passes along the full length of the intestine.

Nonprotein amino acids are not equally abundant in all lakes. In Lake Sibaya, detrital aggregate contains an average of only 4.4 percent total amino acids

(range, 3.5 to 6.0 percent; $N = 12$), 88 percent of which is protein. Combined with low protein levels (*5*), the paucity of nonprotein amino acids in Lake Sibaya detrital aggregate accounts for the stunting of the *S. mossambicus* living there.

These results resolve a long-standing problem concerning the trophic structure of aquatic ecosystems. It has never been clear whether animals that feed on detrital aggregate are microphages that obtain their nutrition exclusively from microorganisms, or are true detritivores (*14*). Since living microorganisms contain negligible amounts of free amino acids, nonprotein amino acids that support the rapid growth of *S. mossambicus* must be present in detritus. This establishes detritivory as a valid trophic category, comparable to carnivory and herbivory.

Finally, these findings raise several interesting questions. What factors influence the abundance of nonprotein detrital amino acids in aquatic systems? Are other detritivorous fish similarly dependent on nonprotein amino acids? Do these compounds play a role in the nutrition of invertebrate detritivores? Answers to these questions will help in the culture of *S. mossambicus* and will contribute to our understanding of aquatic ecosystems.

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References and Notes

1. R. H. Lowe-McConnell, *Fish Communities in Tropical Freshwaters* (Longman, New York, 1975), p. 197.
2. S. H. Bowen, *Nature (London)* **260**, 137 (1976); *Freshwater Biol.* **8**, 449 (1978); M. N. Bruton and B. R. Allanson, *J. Fish. Biol.* **6**, 701 (1974); G. R. Fish, *Uganda J.* **19**, 85 (1955); *Hydrobiologia* **15**, 161 (1960); S. M. Kamal Pasha, *Proc. Indian Acad. Sci. Sect. B* **59**, 340 (1964); G. Nagase, *Z. Vgl. Physiol.* **49**, 270 (1964); T. J. Pandian and P. Raghraman, *Mar. Biol.* **12**, 129 (1972); K. F. Vaas and A. E. Hofstede, *Contrib. Ind. Fish. Res. Stn.* **1**, 1 (1952).
3. S. H. Bowen, *Arch. Hydrobiol.* **87**, 166 (1979).
4. ———, in preparation.
5. ———, *Ecol. Monogr.* **49**, 17 (1979).
6. R. J. Degens, in *Symposium on Organic Matter in Natural Waters*, D. W. Hood, Ed. (Univ. of Alaska, Juneau, 1970), pp. 77–106; G. A. Riley, *Adv. Mar. Biol.* **8**, 1 (1970).
7. W. E. Odum, J. C. Zieman, P. W. Kirk, *Oikos* **32**, 363 (1979).
8. B. B. North, *Limnol. Oceanogr.* **20**, 20 (1975).
9. This was a Durrum D-500 amino acid analyzer in which the ninhydrin reaction is used for detection. Cysteic acid, ornithine, and tryptophan are not detected by the procedure used.
10. N. K. Kauskik and H. B. N. Hynes, *J. Ecol.* **56**, 229 (1968). This technique may overestimate protein in the presence of phenolic compounds or pigment residues [W. D. Loomis, *Methods Enzymol.* **31**, 542 (abstr.) (1974)], but is not known to underestimate protein content.
11. *Nutrient Requirements of Warmwater Fishes* (National Academy of Sciences–National Research Council, Washington, D.C., 1977), p. 11.
12. C. E. Boyd, *Arch. Hydrobiol.* **72**, 1 (1973).
13. To express growth potential, amino acid level is best given as milligrams of assimilable amino acid per kilojoule of assimilable food energy. See Bowen (*5*).
14. C. R. Baier, *Arch. Hydrobiol.* **29**, 183 (1935).

15. I thank R. G. Wetzel, W. M. Lewis, Jr., R. Stones, and I. Railton for helpful comments on the manuscript. The work at Lake Sibaya was supported by South African Council for Scientific and Industrial Research grants to the Institute for Freshwater Studies, Rhodes University, Grahamstown, South Africa. The work at Lake Valencia was supported by NSF grant DEB 76-04300 A01 to W. M. Lewis, Jr., and is part of the joint Venezuelan–North American Lake Valencia Ecosystem Project. Subsequent work was supported by the Graduate School, the Office of Research and Development, the College of Arts and Sciences, and the Department of Biological Sciences at Michigan Technological University. Amino acid analysis was performed by the Department of Biological Chemistry, University of California, Davis.

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Babesia bovis: Continuous Cultivation in a Microaerophilous Stationary Phase Culture

Abstract. *The protozoan parasite* Babesia bovis, *a causative agent of bovine babesiosis, has been continuously cultivated in a settled layer of bovine erythrocytes. Lowered oxygen tension within the layer of host erythrocytes results in a darkening of infected cultures and provides a rapid means of evaluating parasite growth. Deprivation of carbon dioxide causes the merozoites to accumulate in the medium rather than invading new erythrocytes. When separated from the culture, these extraerythrocytic parasites retain their infectivity. Parasites produced in vitro are morphologically identical to parasites from the blood of infected cattle and are susceptible to antibabesial drugs.*

Babesiosis is one of the most important tick-borne diseases of domestic animals: at least 1.3×10^9 domestic animals are at risk worldwide (*1*). Human babesiosis caused by parasites from bovine, rodent, and equine hosts is detected with increasing frequency, indicating the zoonotic potential of these organisms (*2*). Exotic babesias (*3*) occasionally infect animals in the United States. The cattle babesia, *Babesia bovis*, is an obligate intraerythrocytic parasite that occurs in the tropics and subtropics, and its pathogenicity in cattle closely resembles that of *Plasmodium falciparum* in humans. The finding that *P. falciparum*, the causative agent of malignant tertian malaria, could be successfully cultured in vitro (*4*) suggested that the bovine parasite could be cultivated under similar conditions. However, attempts to use the Trager-Jensen method for cultivation of *B. bovis* were unsuccessful (*5*). Short-term cultivation of *B. bovis* was recently accomplished in constantly agitated cultures (*5*), and, by lowering the *p*H to 7, this spinner flask method is reported to support continuous growth (*6*). Relatively low parasite yields (*7*) and the large culture volumes required by this technology limit its application.

Table 1. Statistical data for the microaerophilous stationary phase (MASP) culture of *Babesia bovis*.

Days in culture	82.6
Number of subcultures	33
Parasitemia of 2-day-old subculture*	19.9 ± 7.3
Parasitemia of 3-day-old subculture†	26.1 ± 7.1
Maximum parasitemia	38.1 ± 3.0
Cumulative dilution	2.49×10^{26}
Cumulative increase	1.74×10^{27}
1 log increase‡	73.2 hours

*Mean age, 44.5 hours. †Mean age, 68.6 hours. ‡For routine cultivation, this rate was observed; however, when cultures were subjected to a cumulative dilution of 3.15×10^{17}-fold over 31 days, an average of 42.3 hours was required for a 10-fold increase.

Continuous cultivation of *B. bovis* in a settled layer of bovine erythrocytes has now been achieved, with the parasitemias occasionally exceeding 40 percent. To initiate the cultures we obtain defibrinated peripheral blood from intact or splenectomized *B. bovis*-infected cattle when the parasitemias reach 0.1 to 2 percent (*8*). The use of standard anticoagulants in blood collection, for example, heparin, ethylenediaminetetraacetic acid (EDTA), or acid-citrate-dextrose, is detrimental to the parasites. Infected erythrocytes are suspended to a final packed cell volume of 9.1 percent in a medium consisting of 60 percent Medium 199 (*9*) and 40 percent normal bovine serum (NBS) (*10*), supplemented with 15 m*M* of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 100 μg of streptomycin, and 100 units of penicillin G per milliliter (*11*). The culture suspensions are titrated to *p*H 7 with 1*N* HCl (*12*); portions of the suspensions are then placed in containers in a ratio of 0.62 ml of suspension to 1 cm^2 of culture area. The cultures are incubated at 37° to 38°C under an atmosphere of 5 percent CO_2 and 95 percent humidified air. Every 24 hours the overlying medium is removed and replaced with fresh *Babesia* tissue culture medium No. 4. This medium consists of 40 percent NBS and 60 percent

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