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SOIL NITROGEN INVESTIGATIONS
PAWNEE SITE

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"The quantitative measurement of nitrogen fixation
using the acetylene reduction technique"

INTRODUCTION

Biological nitrogen fixation is brought about largely by free-living bacteria or blue-green algae, which make use of N_2 by non-symbiotic means, and by symbiotic associations composed of a microorganism and a higher plant.

In 1966, R. Schollhorn and R. H. Burris (4), and M. J. Dilworth (1) independently observed inhibition of N_2 fixation by acetylene. Schollhorn and Burris established the competitive nature of this inhibition, and Dilworth found acetylene to be reduced to ethylene in a reaction analogous to the reduction of N_2 to NH_3 . The acetylene reduction technique has analytical advantages of sensitivity, specificity, simplicity, economy, and rapidity over the kjeldahl or ^{15}N methods (3). Biologically, the acetylene reduction technique has advantages in that the characteristics of $C_2H_2 \rightarrow C_2H_4$ parallel those of $N_2 \rightarrow 2NH_3$, it is universal (nitrogenase preparations, and N_2 fixing bacteria, blue-green algae and symbionts reduce C_2H_2 to C_2H_4 , while non- N_2 fixing bacteria do not), the ratio of moles of N_2 fixed to moles of C_2H_4 formed is between 1:3 and 1:4.5, and, finally, the reaction product is specific.

Fixed nitrogen is one of the more important inputs of nitrogen to be considered in the flow of nitrogen through and within a grassland biome. The investigation of biological nitrogen fixation on the Pawnee Site made use of the acetylene reduction technique.

MATERIALS AND METHODS

The acetylene reduction technique for determining N_2 fixation requires specialized equipment. A partial list of this equipment includes:

- Gas chromatograph with hydrogen flame ionization detector

- Gas chromatograph with thermal conductivity detector

- Strip chart potentiometric recorder

- Dual flow controller

- Vacuum pump (mechanical or oil diffusion)

- Vacuum gage

- Manifold system

- Various cylinders of gases with regulators

- Fixation chambers

- Sampling cans

The fixation chambers were constructed in such a manner that a closed system could be maintained. This was done by fusing a 3/8-inch plexiglas base on a 1/4-inch plexiglas cylinder (9 cm in diameter by 25 cm in height) with chloroform. A 3/4-inch flange was fused to the top of the cylinder. The flange contains a machined groove in which an o-ring is seated. A 1/2-inch plexiglas circular end-plate is bolted to the flange by six wing nuts. A gas cock is threaded into the end-plate through which the cylinder is evacuated and the required combinations of gases are admitted. A rubber serum cap is fitted into a hole in the end-plate. Samples of the atmosphere inside the chamber are taken with 1 cc tuberculin syringe through the serum cap.

A manifold system constructed of tygon tubing, gas cocks, and glass tees was connected to a vacuum pump and a mercury manometer. This system was set up so that 6 fixation chambers could be evacuated simultaneously and the required combinations of gases admitted (all by connection to the gas cock located on the top of the chamber).

Undisturbed soil cores were taken by means of a soil storage can from which the bottom had been removed and the edges sharpened. This permits the can to be pushed into the ground, providing soil moisture is adequate. A uniform volume of soil or surface area can be sampled by this method. This method works well on soil-plant cores in which the plant has a fibrous root system. It does not work well with plants having a deep tap root.

After the soil core has been taken and brought into the laboratory, the sampling can and soil core are both placed in the fixation chambers. The diameter of the soil storage can is slightly smaller than that of the fixation chamber. The chamber top is positioned, the chamber evacuated, and the required mixture of gases admitted to the chamber. For aerobic systems, 0.05 atm of acetylene is admitted to the chamber, with the balance to one atmosphere consisting of an 80:20 helium:oxygen mixture. For anaerobic systems, 0.05 atm of acetylene and the balance to one atmosphere of helium is admitted to the chambers. Acetone was removed from the acetylene with a concentrated H_2SO_4 scrubber. Ethylene impurity in the acetylene was negligible.

Samples of the ethylene concentrations in the atmosphere of the chambers are taken periodically with a 1 cc tuberculin syringe. These samples are then injected into the gas chromatograph equipped with a hydrogen flame ionization detector, which is fitted with a 10-ft long, 1/8 inch inside

diameter, stainless steel column containing Porapak R. The oven temperature is maintained at 50°C. The oxygen and nitrogen concentrations are measured by taking similar samples and injecting them into the gas chromatograph equipped with a thermal conductivity detector. This chromatograph is fitted with a five-ft long, 1/4 inch inside diameter stainless steel column containing Molecular Sieve packing.

Standards containing known concentrations of ethylene were made up. Samples of these standards were injected in the chromatograph and resulting peak heights measured. A calibration curve was constructed by plotting moles of ethylene per sample as a function of peak height. A linear plot was obtained allowing computation of a slope factor, (moles ethylene/cm peak height). Quantitative measurements of the ethylene concentrations in the fixation chambers were obtained by measuring the height of the ethylene peak on the chromatogram and multiplying by the slope value obtained from the standards. Rate of N_2 fixation were calculated on the basis of grams $N_2/m^2/day$ or kilograms $N_2/hectare/day$.

Conversion of C_2H_4 formed to N_2 fixed was on the basis of moles of N_2 fixed per 3 moles C_2H_4 .

RESULTS AND DISCUSSION

Preliminary work was performed using alfalfa (*Medicago sativa*) and soybeans (*Glycine max*) that had been grown in a growth chamber. These plants were grown in one-quart ice cream cartons for approximately six weeks. Five soybean plants and several alfalfa plants were contained in the respective cartons. These plants were selected because of their known nitrogen fixing capacity. The alfalfa plants were small enough to be placed

in the chambers without cutting, but part of the aboveground portion had to be removed from the soybean plants. This seemed to have an effect on the duration of the maximum nitrogen fixing rate. The alfalfa plants maintained a straight line accumulation curve, while the soybean N_2 fixation rate fell off after one hour. There appeared to be a lag period of about 15 to 20 minutes before maximum fixation rates were observed. This was probably due to the diffusion of acetylene into the soil-root system and to the diffusion of reduced ethylene back out into the chamber atmosphere.

Fig. 1 shows the accumulation of fixed N_2 by alfalfa and soybeans. The linear portion of the curves indicates a N_2 fixing rate of 150 g/hectare-hour for soybeans and 23 g/hectare/hour for alfalfa. This is equivalent to 3.6 kg N_2 /hectare/day for soybeans and 0.55 kg N_2 /hectare/day for alfalfa. These N_2 fixation rates seem somewhat high for soybeans and possibly a bit low for alfalfa. An excessively high plant population explains the soybean fixation rate, while the fixation rate by alfalfa is underestimated due to small plants and underdeveloped nodules.

Fig. 2 shows the quantity of N_2 fixed under saturated soil moisture conditions by sodded buffalo grass, *Buchloe dactyloides*, cores and by bare soil cores from a temporary lake area. Under these conditions only 1.2 g N_2 /hectare/day were being fixed by the bare lake cores, while the sodded cores were fixing approximately 5 g/hectare/day. The variation in the replications on the sodded cores is likely due to variable microbial populations or energy source rather than analytical error. Actually the disparity is small, considering the low rate of fixation and sensitivity of measurement. Sodded buffalo grass cores at approximately field capacity moisture levels showed only trace amounts of fixation, i.e., < 1 g/hectare/day. It also

should be noted that one set of bare lake soil cores collected earlier showed a higher rate of 10.5 g/hectare/day. Whether this disparity was due to energy source differences or possible blue-green algae accumulation is not known.

Fig. 3 shows the results of adding a soluble energy source to the soil cores to meet the metabolic requirements of the microbial population. This was done by adding a quantity of a 2% sucrose solution which represented 280 kg of actual carbon/hectare. Glucose was also tried, but was less effective than sucrose. The nitrogen-fixing organisms responded to the energy source after approximately an 18-hour lag period. The linear portion of the curves indicate 1 kg of N_2 being fixed/hectare/day for the bare lake soil cores and 0.3 kg of N_2 being fixed/hectare/day for the sodded soil cores. Fairly good replication was obtained in these experiments.

It is not entirely clear at this time whether the decrease in rates, after 80-100 hours where an energy source was added, is due to depletion of the energy source or to inhibition of the enzyme system by acetylene. It should be noted, however, that the magnitude of fixation measured before the rates declined is consistent with published values of N_2 fixed per gram of sugar for pure cultures of N_2 fixing anaerobes. Thus if energy source is the limitation, the addition of the equivalent of 280 kg carbon/hectare resulted in the fixation of less than 1 kg N/hectare on the sodded cores and about 3 kg N/hectare on the lake soil cores.

In the above experiments, the soil cores were saturated with water prior to acetylation. An aerobic atmosphere was maintained within the chamber. This resulted in a partially anaerobic system in the soil. The aerobic atmosphere was maintained by holding the O_2 levels between 10 and 25%. CO_2 produced by root and microbial respiration was trapped in 2 N KOH.

In another set of experiments with bare lake soil cores an anaerobic helium atmosphere was maintained within the chambers. Fixation rates over a 40-hour period indicated about 3.34 g/hectare/day for the check, 0.19 and 1.0 kg/hectare/day using glucose and sucrose respectively. These rates are consistent with the rates measured under saturated moisture conditions.

The range legumes *Astragalus mollissimus* and *Oxytropis lamberti* were observed at weekly intervals throughout the spring and early summer for signs of nodulation, but no nodules were found. Soil cores containing these species were acetylated but no significant N fixation was found. These data must not be regarded as conclusive, as the sampling technique did not work well for cores containing these species. Plants presently are being grown in the greenhouse from seeds collected in the field. These will be examined for nodulation and tested for N fixation by acetylene reduction.

Investigators in Utah recently have found that under conditions of cool temperatures (5 to 15°C) and high moisture levels, a herbaceous sage, *Artemisia ludoviciana*, nodulated profusely (2). Also, reports have been received of possible nodulation of prickly pear, *Opuntia polyacantha*. Examinations of prickly pear and fringed sage, *Artemisia frigida*, have so far failed to show any nodulation. Acetylene reduction measurements on cores at roughly field capacity moisture levels containing these species have shown negligible N fixation.

SUMMARY AND CONCLUSIONS

The acetylene reduction technique was used to measure rates of nitrogen fixation by free-living organisms, presumably bacteria, on soil-plant cores

from the Pawnee Site. At field capacity moisture levels fixation was negligible. Slightly higher rates were observed under saturated or artificially anaerobic conditions. Rates of fixation in these systems ranged from 1 to 5 g/hectare/day. Even the highest figure would represent only 150 g/month. Even in the buffalo grass sod areas, where water collects, the soil is not continually saturated. Also, during a substantial portion of the year, low temperatures severely restrict biological processes. Thus it seems unlikely that more than a few hundred grams/hectare/year are being fixed by this process, while rates on upland areas probably are even lower. Very high levels of energy-supplying material, coupled with anaerobic conditions, result in substantial rates, but the efficiency of conversion is low. On the whole, these data indicate that free-living bacterial fixation is probably not an important source of nitrogen on the grassland.

Nodulation has not been observed on the common range legumes in the area, nor has fixation been detected by the acetylene reduction method. However, observations are limited and the data is not sufficient to draw conclusions concerning the role of symbiotic fixation.

LITERATURE CITED

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Table 1. Summary of nitrogen fixation rate determinations.

| | Saturated Moisture Condition | | Field Moisture (Approx. Field Capacity) |
|----------------------------------|-----------------------------------|---|--|
| | No Energy Source g/hectare/day | Sucrose Added 280 g carbon/ha g/hectare/day | |
| Soybean Plants ^{1/} | 3600 | -- | -- |
| Alfalfa Plants ^{1/} | 550 | -- | -- |
| Sodded Soil Cores | 5.0 | 300 | <1 |
| Bare Lake Cores (1) | 1.2 | 1000 | -- |
| Bare Lake Cores (2) | 10.5 | -- | -- |
| <i>Opuntia polyacantha</i> Cores | -- | -- | <1 |
| <i>Artemisia frigida</i> Cores | -- | -- | <1 |
| Helium Atmosphere | | | |
| Bare Lake Cores | | 1000 | 3.3 |

^{1/} Not from grassland, included for comparative purposes.

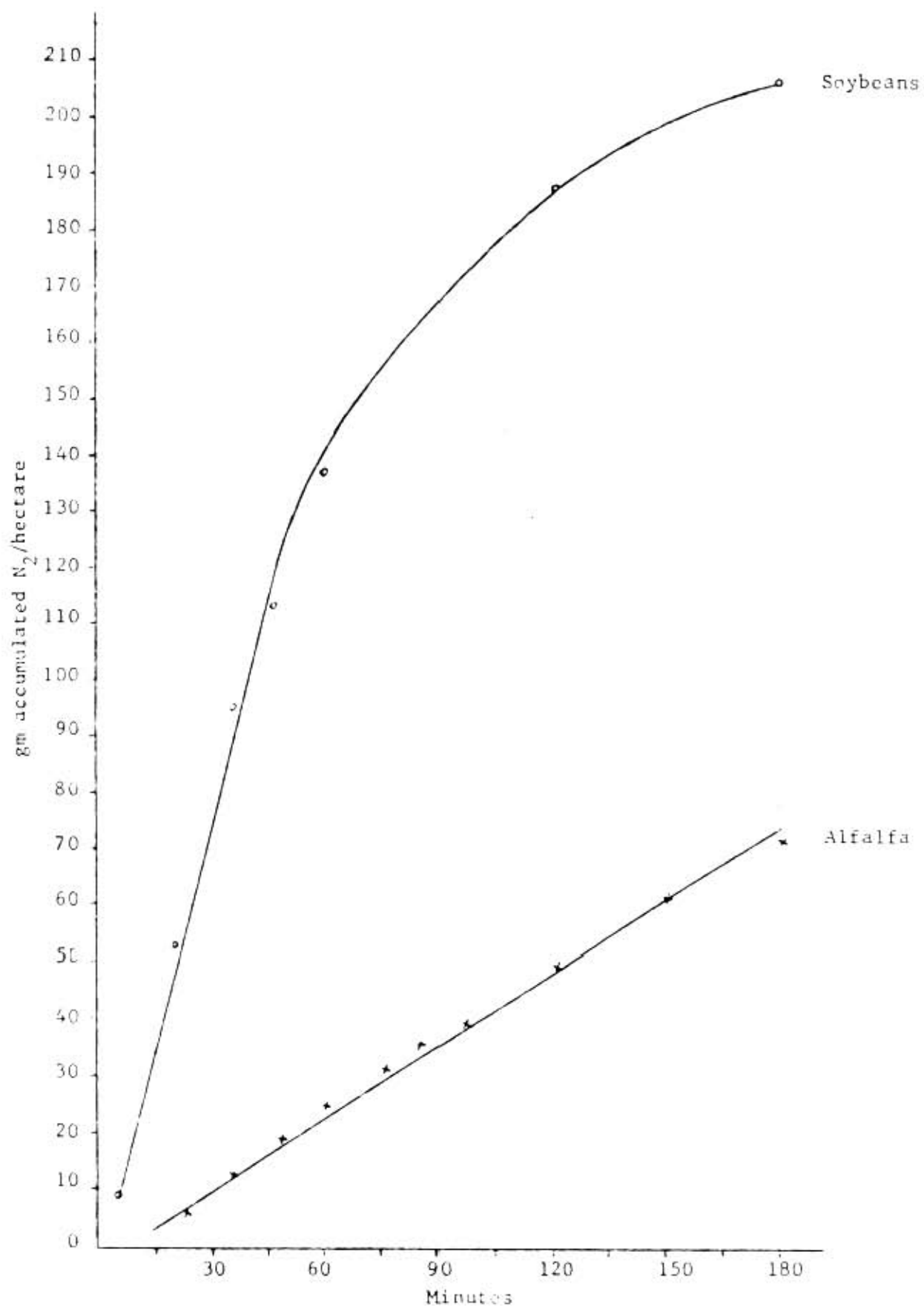


Fig. 1. Accumulated grams of nitrogen fixed/hectare by alfalfa and soybeans.

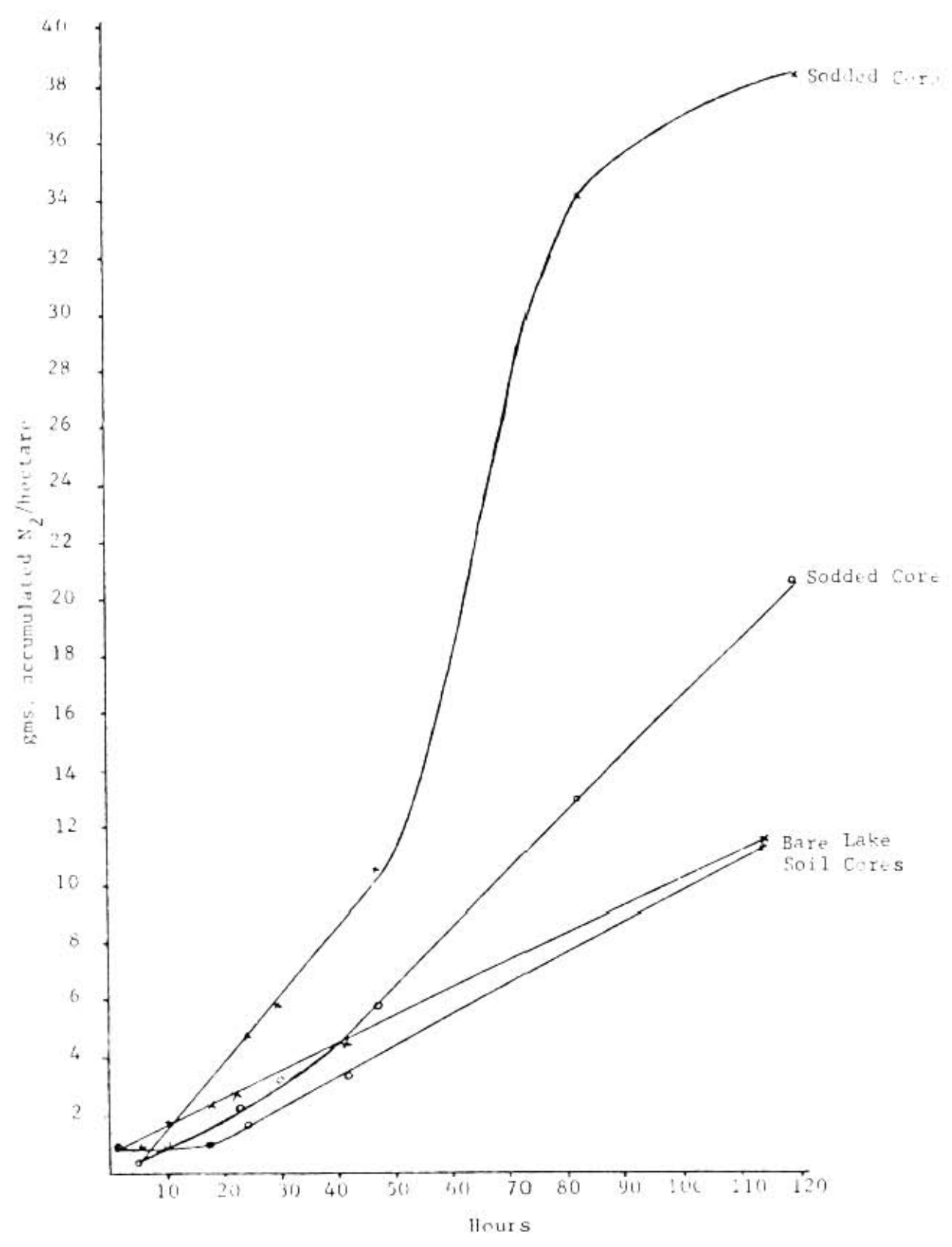


Fig. 2. Accumulated grams of nitrogen fixed/hectare by sodded and bare lake soil cores.

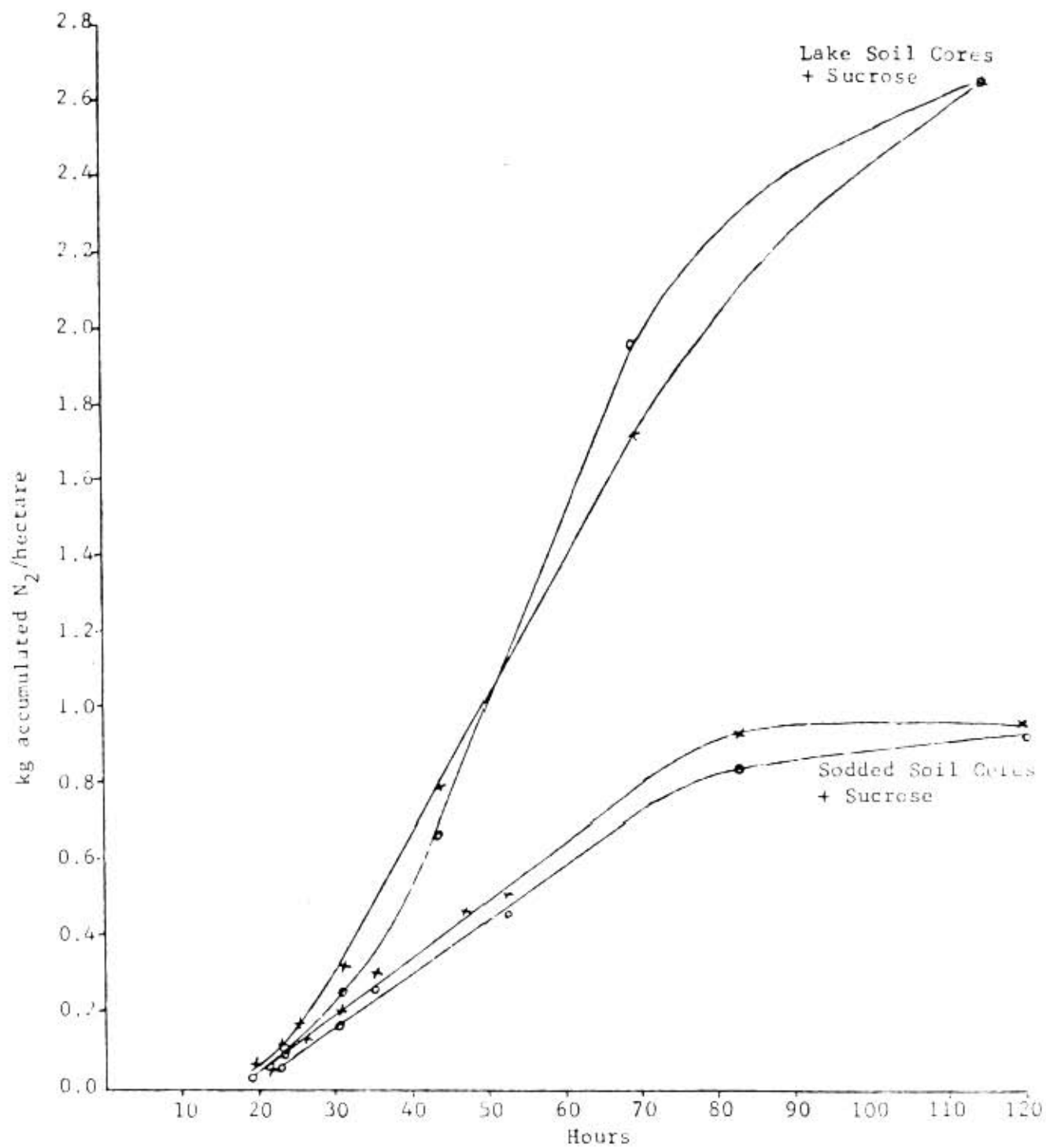


Fig. 3. Accumulated kilograms of nitrogen fixed/hectare by sodded and bare lake soil cores with an energy source added to each.