Plant and Soil **38**, 637–649 (1973) Ms. 2006

ASSESSMENT OF BIOLOGICAL NITROGEN FIXATION IN GRASSLAND AND ASSOCIATED SITES

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SUMMARY

The extent of nitrogen fixation in native grassland on clay soil was measured using the C_2H_2 reduction assay. Undisturbed soil cores incubated in microcanopies in the field indicated fixation rates of 2 kg N/ha per season. Less nitrogen fixation activity was found in associated cultivated soils. Other sites on different soil associations were found capable of fixing 1 kg N/ha or less per growing season. The fixation by several legumes and nodulated nonlegumes ranged up to 1.8 $\mu g/cm^2.h$.

Phyllosphere samples of a moss, *Ceratodon purpureus* Brid reduced appreciable amounts of C_2H_2. An equivalent of 25 to 110 ng N fixation per hour was found per gram of organic material. C_2H_2 reduction in the materials used was related to the availability of light and *Nostoc* spp. were found to be associated with the moss. The major asymbiotic bacteria in the grassland soil were Clostridia although the relationship between numbers of this organism and nitrogen fixation was not significant. *Klebsiella* spp. were isolated and nitrogen fixation measured with C_2H_2 reduction and Kjeldahl methods. Both techniques indicated that the isolated bacteria fixed 30 mg N per litre of media during an 8-day incubation period.

INTRODUCTION

The simplicity, accuracy and economy of the C_2H_2 reduction technique makes routine measurement of nitrogen fixation activity possible. Enough measurements can be made using this technique to obtain adequate replication in field studies, to measure the effect of seasonal variations and to investigate a wide variety of possible nitrogen fixation habitats.

In a previous paper [7] it was reported that nitrogen fixation by a

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Canadian grassland was about 1 kg/ha.year. Similar research [11] indicated that the nitrogen fixing activity of a number of California grassland soils averaged 2,5 kg/ha.year. A Gunnera-Nostoc symbiosis has been reported to produce a positive C_2H_2 reduction test in the phyllosphere [10]. However, other phyllosphere associations have not been adequately tested by *in situ* measurements in the field or by identification of the organisms responsible.

This paper describes an in situ study of the nitrogen fixing capacity, throughout a growing season, of a natural grassland ecosystem, a summerfallow system and other associated sites. Microbiological analyses allowed interpretation of the organisms responsible for nitrogen fixation in the soil cores. Fixation by nodule-bearing plants, nostoc crusts and a phyllosphere system of a moss were investigated.

MATERIALS AND METHODS

Measurement of nitrogen fixation in soil cores

The technique described by Paul *et al.*[7] was used for estimating the rate of nitrogen fixation in undisturbed Sceptre soil cores. Quart size glass jars, in which the glass lid was replaced by a brass or plexiglas top fitted with a 5- or 7-mm serum cap, were used as the microcanopies. After sealing the container, instead of using a special gas mixture, 10 per cent of the air was removed and replaced by C_2H_2. Gas samples were taken with a disposable 1-ml syringe and analysed for C_2H_2 using a Varian 1200 gas chromatograph with a Poropak R column.

Soil samples, 6 cm in diameter by 12.5 cm deep, were taken at random on a virgin grassland site and a summerfallow site. Cores were incubated in the field under conditions as close as possible to those occurring on the original site. Nitrogen fixation was calculated on the basis of measurements of C_2H_4 produced 18 and 24 hours after exposure to C_2H_2.

Small cores, 2 cm in diameter, were used to estimate nitrogen fixation on: a seeded pasture of crested wheat, on a sandy loam soil (Bradwell soil association), a grazed native prairie on a clay soil (Sutherland), and in a second crop of wheat on a calcareous loam in the Weyburn soil association. Exposure to C_2H_2 was similar to that for the Sceptre soils except that 75 ml test tubes were used to enclose the soil core.

The C_2H_2 reduction assay was compared with the N^{15} method in the laboratory with 5 per cent sucrose added to waterlogged soil. The soil was incubated in 25-ml erlenmeyer flasks for 16 days at 15°C. The N^{15} technique used was as reported by Rice and Paul [9]. The numbers of clostridia were estimated in soil samples using the extinction dilution technique of Pochon and Tardieux [8]. Mc Conkey plates and tubes were used for the isolation of klebsiella. Organisms that produced acid and evolved gas were purified and

injected into a culture vessel containing a basal nitrogen-free medium [5]. Anaerobic conditions were maintained in the vessel by removing air and bubbling purified nitrogen through the media continuously. At intervals, samples were taken for growth measurement by optical density, Kjeldahl estimation of nitrogen [2], and the C_2H_2 reduction assay test.

Incubation of 2-g soil samples under waterlogged conditions with the addition of 5 per cent sucrose was carried out to compare anaerobic nitrogen fixation capacity with counts of clostridia in the original field samples. Nodulated root cores were taken by means of a hydraulic coring unit and nitrogen fixation was estimated by the method indicated for the Sceptre soil samples. Subsequently, the nodules were separated from the soil and the weight of organic material in the nodule was determined by dry ashing. Moss and algae were sampled and incubated in 75-ml tubes and C_2H_2 production was estimated similar to the method used for cores, except that a shorter incubation time was used.

RESULTS AND DISCUSSION

Nitrogen fixation in soil cores

Data for the nitrogen fixation activity (measured with C_2H_2), temperature at 5 cm depth and moisture content of the soil cores from the virgin site on the Sceptre clay for the 1971 growing season are presented in Figure 1. Fixation was related to temperature during the early spring period. After the temperature reached 15°C in late May, moisture content had the major effect. The level of fixation dropped during the dry periods at the end of May and July. The calculated value for nitrogen fixation capacity, based on C_2H_2 reduction at various times, during the season was 2 kg N per ha. This was calculated using the conversion factor 3.

The effect of moisture on nitrogen fixation is also reflected by the levels of fixation after irrigation (Table 1). The capacity for nitrogen fixation was measured before irrigation and at intervals after application of water to the virgin Sceptre soil during August. It was also measured in an irrigated plot treated with 186 kg N per ha in the previous year. Fixation activity increased three to four times after one day and remained high until five days after irrigation. The fixation on the plot treated with nitrogen fertilizer in the previous year was lower than that on the plot which was irrigated only.

Although the levels of fixation were increased by irrigation, they did not reach the levels recorded under natural conditions in July (Fig. 1). Separation of the core into different depths showed that the fixation level was high in the 0–1 cm depths and uniformly low at

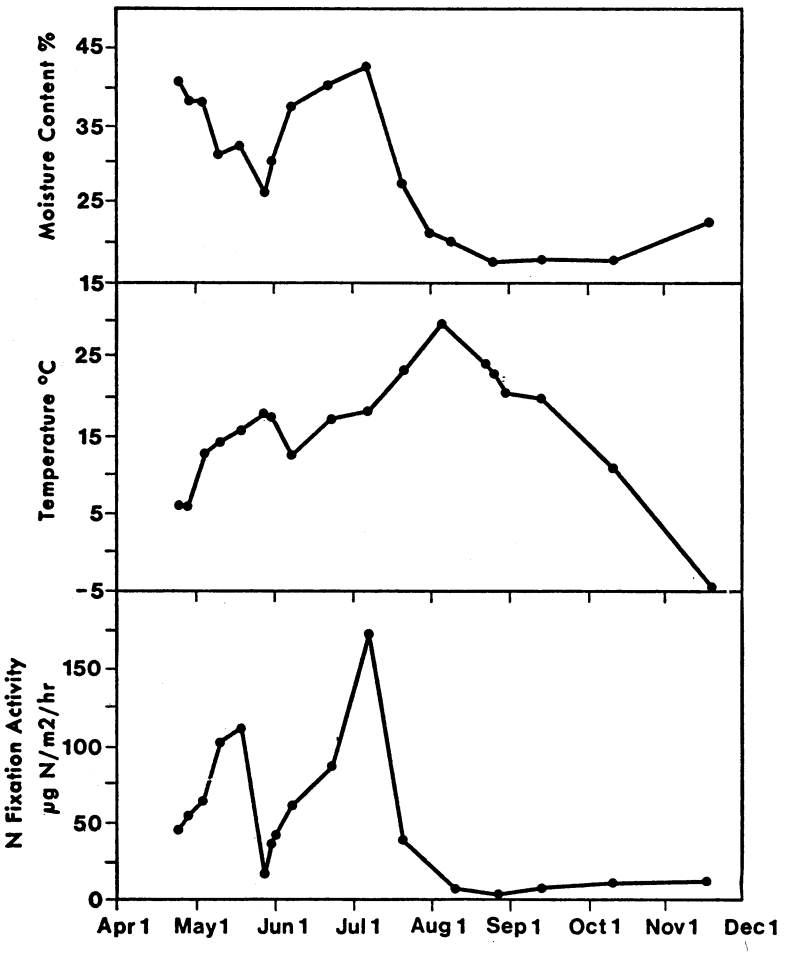

Fig. 1. Percent moisture, soil temperature and nitrogen fixation activity of soil cores from Sceptre clay.

0.06 ng N per g in the 24-cm layer (Table 2). Although the surface has a higher concentration of substrate because of the litter fall, the high levels of activity at this depth also implicate the activity of nitrogen fixing algae which have been found in these soils (R. Cullimore, personal communication).

Fixation activity also was measured on a number of other soils by using 2-cm diameter cores. The data in Table 3 indicate that the Bradwell sandy loam soils showed the highest activity with a calcu-

TABLE 1

Effect of irrigation on nitrogen fixation activity (μg N/m².h)

Plots	Days after irrigation					
	0	1	3	5	7	11
Irrigated	5.3	18.2	n.d.	16.0	6.9	4.5
Irrigated and N-fertilized	3.2	5.3	7.9	n.d.	7.7	n.d.

TABLE 2

Effect of irrigation on nitrogen fixation activity at different depths (ng N/g.h)

Plots	Depths	ng N/g.h
Irrigated	0–1 cm	0.56
	1–12.5 cm	0.06
	12.5–25 cm	0.06
Irrigated and N-fertilized	0–1 cm	0.23
	1–12.5 cm	0.06
	12.5–25 cm	0.07

TABLE 3

Nitrogen fixation activity of small core samples from the Saskatoon area

Sampling date	Bradwell soil		Sutherland soil		Weyburn soil	
	μg N/m².h	% M*	μg N/m².h	% M*	μg N/m².h	% M*
April 14	54	22.9	—	—	—	—
May 5	12	18.6	7	34.9	—	—
May 20	12	9.5	1	24.0	—	—
June 1	8	12.0	8	29.4	32	28.0
July 5	16	18.7	12	26.5	5	25.8
July 22	93	17.2	0	29.3	5	15.8
Sept. 1	37	12.7	—	—	0	23.4

* M = moisture content.

lated total of 1 kg N per ha fixed during the growing season. This was more than twice the amount observed in the grazed native prairie on Sutherland clay and the wheat field on Weyburn loam. Table 3 also indicates the relation between moisture content and nitrogen fixation activity over the season on the sites.

Correlation coefficients and simple regression equations were calculated between nitrogen fixation activity and temperature and moisture. Some data reported by Paul *et al.*[7] are included for the 1970 growing season on the Sceptre grassland site (Table 4). The regression for different combinations of the available data indicate the close relation between soil moisture and nitrogen fixing capacity. The r values are negative due to the conversion of moisture content to pF values determined from moisture retention curves for the different soils [1 6]. The use of all soils investigated in 1971 gave a low correlation coefficient with only a small percentage of the variability in nitrogen fixation being accounted for by moisture ($r = -0.39$). The virgin site on the Sceptre clay alone gave a higher correlation coefficient ($r = -0.73$). In the field over the growing season, no significant relationship was found between, nitrogen fixation activity and temperature, although the temperature strongly affects nitrogen fixation early in spring as shown in Figure 1.

Elimination of the values for early spring in the virgin site increased the correlation coefficient ($r = -0.81$). It was considered that there were not enough data for an adequate multiple regression relating the effects of moisture and temperature. The differences in the equation for 1970 and 1971 corroborate the earlier observations on the effect of irrigation (Table 1) that factors other than H_2O have a significant effect on fixation. Although the correlation coefficients for the two years were highly significant, different parameters for the equations were obtained.

Microbiological analysis

The effect of microbial populations on nitrogen fixation in the field was studied in two ways. The first of these involved the addition of 5 per cent glucose and incubation of the clay soils under waterlogged conditions for 24 hours during which time the C_2H_2 reduction was measured (fixation potential). The second approach involved attempts to correlate fixation in the field with total microbial counts, growth on nitrogen-free media, and McConkey agar and extinction dilution counts of nitrogen fixing clostridia and algae. No relationships were found between fixation and total counts, growth on nitrogen-free media, McConkey agar or the algal estimates. The fixation potential did vary with the field measurements of the virgin plots

TABLE 4

Correlation coefficients and equations between nitrogen fixation activity and moisture content in different soils

Soil type	r	equations
Sceptre clay, 1970 growing season	−0.596*	y = 94.8 − 23.0x
Sceptre clay, 1971 growing season	−0.728**	y = 218.4 − 47.6x
Sceptre clay, 1971 (above 15°C)	−0.807**	y = 259.2 − 57.5x
All investigated soils, 1971	−0.394**	y = 109.3 − 24.1x

* Significant at $P = 0.05$.
** Significant at $P = 0.01$.
y = nitrogen fixation activity (μg N/m² per h).
x = moisture content as pF.

TABLE 5

Microbial counts and nitrogen fixation activity of Sceptre clay soils

Sampling date	Log Clos. No.'s	Fixation ng N/g.h	Fixation potential ng N/g.h
Virgin plot			
April 28	1.60	0.55	16
May 11	3.10	1.03	23
May 18	2.78	1.10	33
May 26	2.51	0.15	12
June 1	2.34	0.38	15
June 8	2.53	0.73	28
July 20	3.80	0.40	21
Aug. 4	2.33	0.05	
Aug. 25	4.04	0.25	
Sept. 14	2.31	0.08	
Cultivated plot			
April 28	4.55	0.02	
May 4	3.54	0	
May 18	3.84	0.02	
June 1	2.80	—	
July 20	2.68	0.07	
Aug. 25	4.60	0.05	
Sept. 14	2.81	0.03	

(Table 5), r = 0.84) even though the potential was approximately 30 times as high as the actual levels found in the field.

Another organism isolated from the clay soil under study showed significant fixation rates in nitrogen-free media under anaerobic conditions. There was a day to day variation in optical density and

differences in Kjeldahl nitrogen and the C_2H_2 reduction rate during the 8-day period (Table 6), with a lag in C_2H_2 reduction. However, over the 8-day incubation the activity calculated on a 3:1 basis for C_2H_2 reduction relative to N_2 fixation equalled the increase of total N in the system. The organism responsible for the nitrogen fixation was judged to belong to the Klebsiella group on the basis of the gram stain, acid production, gas development, small cell size and nitrogen fixing capacity under anaerobic conditions.

Comparison of the Kjeldahl method, N^{15} and the C_2H_2 reduction assay in small waterlogged soil-glucose samples similar to those used for the estimation of nitrogen fixation potential gave a C_2H_2 to $N_2{}^{15}$ reduction of ratio 2.6 (Table 7). The data differ from waterlogged experiments reported by Rice and Paul [9] in that there was a larger surface exposed to the gas phase in this experiment and the content of N_2 in the N^{15} systems was 20 per cent instead of 10 per cent. The

TABLE 6

Growth and nitrogen fixation by an anaerobic culture

Day	Optical density change per day	mg N/day.litre	
		Acetylene reduction	Kjeldahl
0–3	0.010	0.70	1.60
3–4	0.030	1.70	2.60
4–5	0.163	1.85	5.90
5–6	0.105	4.35	4.10
6–7	0.085	4.60	2.55
7–8	0.030	1.90	1.20
8–9	−0.025	1.00	0.10

TABLE 7

Comparison of methods for studying nitrogen fixation in a waterlogged soil-glucose system at 15°C during 16 days incubation period

Method	μM fixed per g soil
Kjeldahl method	11.6
N^{15} method	14.4
Acetylene reduction assay	17.3*

* Conversion factor 3 for C_2H_2 : H_2.

TABLE 8

Nitrogen fixation in soil cores from nodulated plants

Plant species	μg N/g per h	mg N/g nod. per day
Astragalus spp.	54	1.3
Caragana arborescens	335	8.0
Medicago sativa	322	7.7
Trifolium agrarium	415	10.0
Elaeagnus commutata	143	3.4
Shepherdia argentea	92	2.2

TABLE 9

Nitrogen fixation in phyllosphere samples

Sample	ng N/g dry wt per hr
Ceratodon purpureus Brid	146
Unidentified moss	159
Unidentified moss	46
Lichen from Matador site	122

exposure of soils amended with cellulose, to C_2H_2 indicated an inhibitory effect of C_2H_2 on both total numbers and on cellulose decomposition (data not shown). This is in agreement with earlier observations by Rice and Paul [9], and Brouzes and Knowles [3], that C_2H_2 has an inhibitory effect on certain microbial growth parameters. This inhibitory effect must be taken into account in experiments utilizing long-term exposure to this gas.

Nitrogen fixation in associated sites

Symbiotic nitrogen fixation in nodule-bearing plants such as: *Astragalus striatus, Caragana arborescens, Medicago sativa, Trifolium agrarium, Elaeagnus commutata,* and *Shepherdia argentea* was studied both on the basis of nodule weight and undisturbed soil cores (Table 8). A maximum rate of 400 μg N per g per hour was found in the leguminous plants. The non-legumes *Elaeagnus commutata* (wolf-willow) and *Shepherdia argentea* (buffalo berry) had fixation rates of $\frac{1}{3}$ of $\frac{1}{4}$ of those of the more active legumes. Because of the dispersed distribution of symbiotic systems under virgin conditions and the lack of information on the weight of active nodules per unit area, it

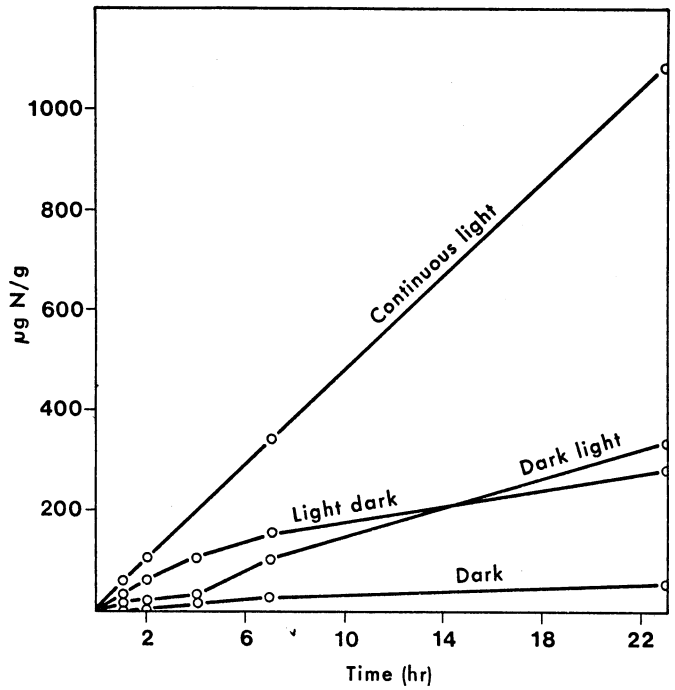

Fig. 2. Nitrogen fixation activity of *Nostoc* sp. incubated in the light and dark after preincubation.

is difficult to extrapolate data such as these to field conditions. However, estimates during 1971 indicated a fixation level of 37 mg per m^2 per day of *Elaeagnus* habitat. Measurements in June 1972 indicated 136.4 mg per m^2 per day for *Shepherdia* and 17.4 mg per m^2 per day for *Elaeagnus*.

Fixation by blue-green algae

Cores of grassland plots containing Nostoc crusts or moss were found to consistently fix more nitrogen than cores without these organisms. The effect of light on samples of *Nostoc* sp. which appear as crusts on the surface of many areas of the grassland soils examined is shown in Figure 2. The graph shows the data for a 22-hour exposure period after 15 hours of preincubation in either the light or the dark. The straight line in continuous light indicates a fixation capacity of 50 µg N per g of material per hour. This value is very

close to that reported earlier for similar crusts grown under optimum moisture conditions [7]. There was a small amount of fixation for 4 hours after preincubation in the light and then exposure to C_2H_2 in the dark as shown by the light–dark curve. A slight lag upon exposure to the light of samples preincubated in the dark also was noted.

Moss samples of *Ceratodon purpureus* Brid were found capable of fixing 25 to 110 ng N per g dry matter per hour depending upon the moisture content (data not shown). Thus, fixation in this phyllosphere association was $^1/_{500}$ that of the Nostoc crusts. Fixation was associated with the leaves of the mosses. Blue-green algae were microscopically observed to be associated with the mosses. However, the possibility of bacterial fixation could not be discounted. Attempts to wash the organisms off the leaves did not result in any C_2H_2 reduction activity in the wash water. But the activity of the leaves increased probably due to increased moisture content after washing. Determination of the site of fixation showed that 98 per cent of the fixation of the total plants appeared above ground with fixation being sensitive to light. Preincubation for 15 hours in the light and then exposure to C_2H_2 under various conditions showed data very similar to that previously observed for Nostoc except that the values for the moss-microbial association as would be expected were much lower than pure Nostoc (Fig. 3).

Determination of the algae on the above ground portions of moss from a number of sites indicated a variety of organisms including Nostoc, Lyngbya, Chlorella, Pinnularis, Diatoms, Spongiochloris, Oscillatoria, Anabeana, and Mesotaenium. Nostoc was the only organism common to all sites and occurred in concentrations of 500 cells per gram on a wet site and 250 cells per gram on a drier site (R. Cullimore, private communication). The demonstration of active fixation in *Ceratodon purpureus*, a very common moss in both upland and moist areas in the grassland, especially on areas of low vegetation and organic matter such as riverbanks and the association of Nostoc which raises the question of the importance of such associations in the initial development of organic matter on many sites. Algal associations with mosses have been demonstrated previously [12] but little or nothing is known of the nitrogen fixation activity of these associations.

The use of the C_2H_2 assay confirmed by N^{15} and Kjeldahl tech-

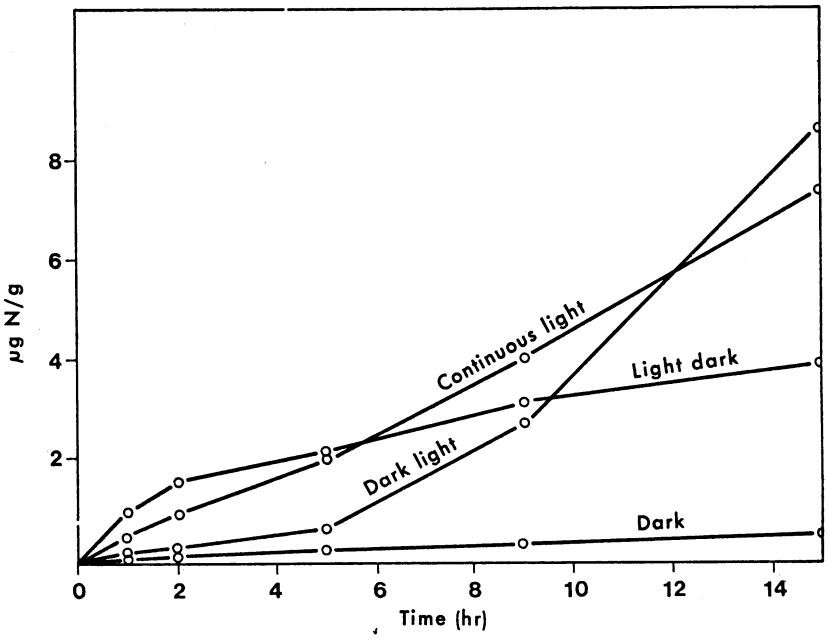

Fig. 3. Nitrogen fixation activity of moss samples incubated in the light and dark after preincubation.

niques has made possible a survey of the nitrogen fixation potential of a number of grassland microbial plant associations. The legume and non-legume symbiotic fixation rates corroborate previous estimates of the significance of these plants where they occur. Integration of the measurements of non-symbiotic organisms over a complete growing season also made possible an assessment of the significance of non-symbiotic fixation and showed its high dependence on moisture availability.

The moss–algal relationship is of special interest. Availability of the C_2H_2 technique means that it should not be difficult to ascertain the significance of this and other associations which no doubt will be found in nature.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. R. Cullimore for identification and counting of the algae associated with the mosses. Issued as Saskatchewan Institute of Pedology No. R 103.

A portion of this research was conducted under the auspices of the Matador I.B.P. project and is issued as C.C.I.B.P. No. 146.

Received July 4, 1972

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