N^{15} nitrogen gas was used. The plants were harvested after 56 days and analyzed. The experimental results are given in Table 1 as atom per cent. N^{15} excess of plants over the average values of air control plants.

TABLE 1

	Atom per cent. N^{15} excess of plants over average of air controls	
	Exp. 1	Exp. 2
Bacteria-free barley	-0.010 ± 0.005*	-0.006 ± 0.005
Bacteria-free clover	-0.004 ± 0.005	
Inoculated clover	2.469 ± 0.061	0.689 ± 0.052

* 0.005 per cent. represents the standard deviation of spectrometer readings for all determinations on the bacteria-free plants; the standard deviations of readings for the inoculated clover (calculated for individual samples) are higher, since the error of measurement is greater at higher N^{15} concentrations. The regular occurrence of negative values for bacteria-free plants is merely fortuitous.

These data show that if either barley or bacteria-free clover fixed any nitrogen, the amount fixed was within experimental error, whereas fixation by inoculated clover resulted in the accumulation of large quantities of N^{15}.

By calculation from the data of Ruben *et al.*,[1] we can find if their success and our failure to observe fixation arises from a difference in the sensitivity of the stable and radioactive tracer methods. These investigators used 30 grams wet weight of barley tops which, assuming 75 per cent. moisture and 3 per cent. nitrogen on a dry weight basis, would contain about 225 mg of nitrogen. The authors stated that during the experiment the plants assimilated an amount of N_2 which "corresponds roughly to 0.01 cc of N_2"; 0.01 cc of N_2 (0.0125 mg N_2) constitutes 0.00556 per cent. of the total nitrogen of the plants. We can calculate what the final N^{15} content of our barley plants should be if we assume that the rate of fixation reported by Ruben *et al.*[1] occurred uniformly during the period of our experiment 1. Since 42 days' exposure is 3,024 times the 20-minute treatment of Ruben *et al.*,[1] 16.8 per cent. (*i.e.*, 0.00556 per cent. $\times$ 3,024) of the total nitrogen of the plants would be fixed during the experimental period. But as 13.5 atom per cent. excess N^{15} was used, the observed N^{15} value would be 2.27 atom per cent. N^{15} excess (*i.e.*, 16.8 per cent. $\times$ 0.135).

The value 2.27 atom per cent. N^{15} excess, which we would have found had our barley plants fixed nitrogen at the same rate as Ruben *et al.*[1] report for N^{13} fixation, is 454 times the standard deviation of our measurements with the mass spectrometer. It is about the same value as we actually observed with inoculated clover plants.

Since the fixation experiments with barley were completely negative, one can but speculate as to the reason that the twenty-minute exposure of excised barley tops in the experiments of Ruben *et al.*[1] resulted in an uptake of N^{13}. These workers extracted the barley plants with boiling 80 per cent. ethyl alcohol, boiled the extract in a stream of air in an effort to drive off N_2, and then detected radioactivity in the boiled extract. It hardly seems likely that the observed N^{13} uptake can be attributed to fixation by the small number of bacteria carried by the plant tops or, in view of our results, to a true nitrogen fixation by the plant tops themselves. Ruben *et al.*[1] state, "These experiments with N^{13} do not necessarily prove that a net uptake of N_2 has occurred, since the existence of reversible (interchange) reactions involving N_2 is possible." However, Burris and Miller[4] demonstrated the absence of any interchange reaction in *Azotobacter vinelandii*, which was vigorously fixing N_2 in a non-equilibrium N^{15}-excess atmosphere. The possibility remains that a non-specific surface adsorption of N_2 by fresh barley tops and a failure to completely remove this N_2 containing N^{13} accounts for the radioactivity detected by Ruben *et al.*[1] In our experiments it is obvious that the Kjeldahl treatment would eliminate all adsorbed N_2.

The complete lack of fixation of the stable N^{15} isotope by bacteria-free barley and bacteria-free red clover plants under conditions identical with those supporting active fixation of N^{15} by red clover inoculated with *R. trifolii* supports the generally accepted conclusion that non-leguminous plants and leguminous plants in the absence of the root-nodule bacteria are unable to fix molecular nitrogen.

In addition to the question of nitrogen fixation by non-leguminous plants, positive and negative reports in the literature present controversies concerning the nitrogen fixing ability of germinating pea seeds, excised root nodules with added oxalacetic acid, and root nodule bacteria in the absence of the host plant. Thus far we have been unable to demonstrate fixation of N^{15} by any of these biological agents, whereas azotobacter and leguminous plants with root nodule bacteria fix N^{15} readily.

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CROWN GALL PRODUCTION BY BACTERIA-FREE TUMOR TISSUES

CROWN gall has in the past been produced only by inoculation of a host plant with *Phytomonas tumefaciens* (Smith and Town.) Bergey *et al.* either as a pure culture or in the form of a preparation of tissues infected therewith. Although crown-gall tissues have not always yielded cultures of the organism, it has been presumed that the bacteria were present or at least had been present at some stage in the development of the tumor. The production of tumors with-

[4] R. H. Burris and C. E. Miller, SCIENCE, 93: 114, 1941.
[5] National Research Council fellow.

out direct stimulation from the bacteria, although envisioned as a possibility by certain workers, has not previously been demonstrated, nor has that possibility been given serious credence by other investigators.

One of us has recently confirmed the work of Smith *et al.*,[1] showing that true secondary tumors developed in sunflower plants at a distance of several internodes from the site of the primary tumor, even when inoculations were made into fully elongated internodes at a distance from the apical bud.[2] Many of the secondary tumors were found to be sterile by the usual bacteriological criteria. The bacteria-free secondary tumors appeared, therefore, to be admirably suited for an experimental study of the question as to whether host cells under the influence of the bacteria acquire the capacity for autonomous growth.

Recently developed techniques for the *in vitro* cultivation of excised plant tissues[3] were applied to the study of secondary tumors. Tissue fragments from the interior of a series of such tumors were removed aseptically and placed in 125 ml Erlenmeyer flasks, each containing 50 ml of White's standard glycine-thiamin nutrient[4] stiffened with 0.6 per cent. of thoroughly leached agar. Out of 107 original isolations from tumors, 2 were contaminated with bacteria and 4 with molds. Out of 50 control cultures taken from healthy plants, 4 were contaminated with bacteria and 3 with molds. The larger tissue masses available in the tumors and the consequent greater ease of manipulation were doubtless responsible for the lower incidence of contaminations in tumor cultures.

Cultures capable of continued rapid growth were not established from any of the small petiolar tumors nor from the petioles of healthy plants. On the other hand, out of 37 isolations from large secondary tumors from the stems at nodes, 19 grew successfully. Six isolations were carried through 3 or more passages, being divided at each passage. Two strains, one isolated on February 10, 1941, the other on March 25, 1941, were retained for detailed study. The first of these isolations has, at the time of writing (August, 1941), been maintained through 13 successive passages, has been divided into 482 pieces, each several times as large as the original, and has undergone a theoretical increase[5, 3] in volume of approximately 450,000 times. Throughout this manipulation, it has been constantly in contact, both at intact and at freshly cut surfaces, with a nutrient shown by ourselves and by others to be capable of supporting a profuse growth of *Phytomonas tumefaciens;* yet, out of the 482 cultures not a single one has developed any bacterial growth. Attempts to isolate bacteria from these cultures by grinding and plating on nutrient agar or in broth have consistently failed. When cultures were ground and the paste injected into sunflower or tomato plants, no galls were produced such as regularly appeared when a paste prepared in a similar manner from young primary tumors was injected. The results seem to furnish almost unquestionable evidence that these tissues, which multiply rapidly, nevertheless do so without continued stimulation from crown-gall bacteria. Their capacity for autonomous growth appears established.

Tumor tissues grown *in vitro* are colorless or, especially just after being divided, slightly tinged with brown from oxidative products, scattered remnants of dead cells, etc. The surfaces are rough, covered with irregular unorganized protuberances, and may put forth unorganized outgrowths either along the agar or up into the air. They are firm but easily cut and of uniform texture. Histologically they are mostly parenchymatous with scattered scalariform elements. These elements form a structure closely resembling that of crown-gall tissue stimulated by *Phytomonas tumefaciens* when inoculated into parenchymatous tissues of the host.

Tissues from cambial and procambial regions of healthy sunflowers have likewise been isolated in culture but present a very different picture. In the 9 weeks that they have been maintained, they have been carried through 5 passages, but their growth rate has been so slow that the volume increase during this period has been of the order of 30 times as compared to about 400 times for the gall tissues over a corresponding period. Cultures of normal tissue maintain their outlines, whereas gall cultures grow as irregular or subglobular masses. Normal tissue cultures regularly contain chlorophyll, are woody in texture and frequently produce roots, a phenomenon not observed to date in cultures of gall tissues.

At the end of 5 successive passages *in vitro,* 10 tumor cultures were grafted back into young healthy sunflower plants. Of these, 5 implants had, at the end of 7 weeks, grown into typical crown-gall tumors having diameters up to 1 cm, one plant was accidentally broken, and 4 implants failed to grow. Similar results have been obtained with a second series of grafts using cultures from the 6th passage and a third series using cultures from the 10th passage. Attempts to isolate bacteria from one of the tumors by grinding and plating in nutrient dextrose agar failed. The results seem to indicate that the tumor-inducing capacity has been retained by these bacteria-free tissues through at least 10 successive passages *in*

[1] E. F. Smith, N. A. Brown and L. McCulloch, *U. S. Dept. Agr., Bur. Pl. Ind., Bull.* 255, 1912.
[2] A. C. Broun, *Phytopath.,* 31: 135–149, 1941.
[3] P. R. White, *Am. Jour. Bot.,* 26: 59–64, 1939.
[4] P. R. White, *Plant Physiol.,* 14: 527–538, 1939.
[5] P. R. White, *Plant Physiol.,* 9: 585–600, 1934.

vitro. Preliminary examination has shown the induced tumors to have a histological structure considerably more uniform than that of most crown galls, with extensive hyperplasia but relatively little disorganization. This last feature is quite marked and may possibly be due to the uniform distribution of the mechanism of hyperplasia in contrast to the scattered and localized centers of stimulus characteristic of bacterial galls. Fusion with the host tissue was excellent, although growth was mostly if not entirely a function of the transplant, as is the case with transplanted animal neoplasms. It is clear from these results that the affected tissues have undergone a drastic change which is indicated, first, by their capacity to produce galls, a quality not found in normal tissue, and, second, by their behavior *in vitro*, where their growth habits differ markedly from those of normal tissues under identical conditions. That this change was originally brought about by some stimulus from the crown gall organism seems clear. That its maintenance is not dependent on the continued presence of the bacteria is equally clear. Further details will be published elsewhere.

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COLLODION FIXATION: A NEW IMMUNOLOGICAL REACTION

THE study of filterable viruses and of diseases caused by them is handicapped by the relative lack of *in vitro* reactions which can detect very small amounts of virus substance. The present dependence on pathological processes for the identification of viruses involves a considerable time factor. As an approach to the solution of these difficulties experiments were undertaken with a well-known immunological system in an effort to increase sensitivity, since this would appear to be the first requirement of any new method. The system employed was the reaction between antipneumococcus serum and the specific capsular polysaccharide of the pneumococcus. Under optimum conditions, specific precipitin reactions can be obtained with polysaccharide dilutions as high as 1–5,000,000; with complement fixation to 1–20,000,000.

The first series of experiments involved the adsorption of *antibody* on the surface of collodion particles, thereby artificially increasing the size of the antibody. Many workers have adsorbed *antigen* on collodion or other particles (*cf.* review by Cannon and Marshall[1]) and have demonstrated increased sensitivity as regards detection of antibody. Cannon and Marshall[1] sensitized collodion pellets with egg albumin; Weir[2] used tuberculin sensitized pellets for the study of antibody formation in animals. Our experiments in the adsorption of antibody were successful under very limited conditions; the most fortunate results were obtained with the use of purified horse and rabbit antipneumococcus sera. Particles so sensitized were agglutinated in the presence of the specific capsular polysaccharide in dilutions of approximately 10^{-10}. The details of these experiments will be published elsewhere. It may be said, however, that these systems are very sensitive to non-specific factors, such as broth, proteins, changes in electrolyte concentration, etc., and hence are not suitable for the general purposes of the method most desired.

In an approach to a more satisfactory method it was recalled that although both antigens and antibodies are not remarkable for adsorptive phenomena, the antigen-antibody complex is extraordinary in this respect: the complement fixation reaction is an example of this property.[3] Experiments were therefore undertaken to learn whether the antigen-antibody complex would adsorb collodion particles, thus, as it were, magnifying immunological reactions.

Collodion particles were prepared after the method of Cannon and Marshall,[1] the stock suspension being adjusted to a density such that a 1–10 dilution would match number 3 on the McFarland scale.[4] Suitable and constant quantities of collodion suspension are added to varying amounts of antigen in agglutination tubes. To these mixtures are then added appropriate amounts of immune sera; the total volume is then brought to 1.0 cc by the addition of physiological saline. With systems of some refinement, such as that with antipneumococcus serum, the tubes remain at room temperature for one hour and are then centrifuged for 5 minutes at 500 r.p.m. Each tube is "flipped" and the amount of particulate agglutination estimated. Control tubes, not containing antigen, give free and smooth resuspension. With less refined systems, such as the viruses, the tubes are placed in the icebox overnight, then centrifuged and read. An example of results obtained with the antipneumococcus system is given in Table I.

These results present two important points: (a) The

[1] P. R. Cannon and C. E. Marshall, *Jour. Immunol.*, 38: 365, 1940.

[2] J. M. Weir, *Proc. Soc. Exper. Biol. and Med.*, 46: 47, 1941.

[3] K. Goodner and F. L. Horsfall, Jr., *Jour. Exper. Med.*, 64: 201, 1936.

[4] J. McFarland, *Jour. Am. Med. Assn.*, 49: 1176, 1907.