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Evidence for the Presence of Bacteria-specific Proteins in Sterile Crown Gall Tumor Tissue[1]

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ABSTRACT

Cross-reacting antigens were found in bacteria-free crown gall tumor tissue tested with serum prepared against *Agrobacterium tumefaciens* (Smith and Towns.) Conn., but no such antigens were detected in callus tissue. Soluble proteins from tumor tissue, callus tissue, and the crown gall bacteria were fractionated on a DEAE-Sephadex (A-50) column. The diethylaminoethyl-Sephadex elution profile for tumor tissue showed three protein fractions that were not detected in the callus tissue. Two of these protein fractions were shown to be exclusively bacteria specific. Besides these qualitative differences between the two tissues, significant quantitative differences in the amount of protein fractions were also observed. The diethylaminoethyl-Sephadex column fractions from tumorigenic strain of *A. tumefaciens* corresponding in position to the three additional peaks in the tumor tissue also showed cross-reacting antigens when tested with serum prepared against sterile tumor tissue. It is suggested that tumor formation by *A. tumefaciens* involves integration of the bacterial genome into the host-cell genome.

Normal plant cells, on transformation into tumor cells by the crown gall organism (*Agrobacterium tumefaciens* [Smith and Towns.] Conn.), acquire the ability to synthesize auxin, cytokinin, and other substances in abundance, substances that must be supplied to normal cells for growth *in vitro* (3). Increases in RNA synthesis (11) and in the activities of certain enzymes (13), as well as the production of new enzyme proteins (8), also occur in transformed cells. These modifications could occur either by a persistent de-repression of a segment of the normal cell genome or by the incorporation of a self-replicating foreign genome into the host genome. No significant differences between normal and crown gall tumor tissue were found in the properties of the chromatin, histone, or nonhistone chromatin proteins (12). On the other hand, studies in this laboratory (6) and elsewhere (7) suggest that crown gall tumor formation may involve integration of the bacterial genome with the host genome. Srivastava and Chadha (14) have recently demonstrated the liberation of *A. tumefaciens* DNA from crown gall tumor-cell DNA on shearing. Since bacteria-specific DNA (7) and RNA (6) have been detected in the tumor tissue, we investigated whether the tumor tissue also contained bacteria-specific proteins that could be detected by highly specific immunological procedures and purified by chromatographic methods. The results of this investigation are reported in this paper.

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MATERIALS AND METHODS

Cultures of normal tissue and bacteria-free crown gall tumor tissue of tobacco (*Nicotiana tabacum* L. var. Wisconsin 38), maintained on complete and minus IAA and kinetin media, respectively (11), were used in the present investigation. Electron microscope examination of the tumor tissue by Dr. R. Ziegel of this institute revealed no bacteria, mycoplasma, or virus-like particles. *A. tumefaciens* strains 4-32 (tumorigenic) and 11 NV6 (nontumorigenic) were maintained on slants of nutrient agar containing 0.5% sodium chloride (w/v). When needed, bacteria were grown for 48 hr at 28 C in nutrient broth containing 0.5% sodium chloride as shake cultures. Bacterial cells were harvested by low speed centrifugation in the cold.

Protein Extraction from Plant Tissue. At the end of 28 days of culture, 5 g of tissue was extracted with a mortar and pestle into medium A (50 mM tris-HCl and 5 mM L-cysteine, pH 7.4) containing 20 mM β-mercaptoethanol and 0.1 mM Cleland's reagent. For each gram of tissue, 5 ml of the extraction buffer was used. Also, 0.5 g of acid-washed sand and 2.0 g of insoluble polyvinyl pyrrolidone (Polyclar-AT powder, General Aniline Film Corporation, N. Y.), previously equilibrated (5) with the extraction buffer, were added at the time of grinding. The homogenate was centrifuged at 30,000*g* for 30 min. Supernatant I was saved, and the pellet obtained was washed two or three times with the extraction buffer to ensure complete harvest of soluble proteins. Protein estimations of the supernatants from a series of washings revealed that an average of 90% of the soluble proteins were recovered by this procedure.

Supernatants I, II, and III (30–35 ml) were combined, and were dialyzed at 1 C for 24 to 36 hr against medium A, with frequent changes. The dialyzed extract was clarified by centrifugation at 30,000*g* for 15 min. This clarified extract was either used for chromatography on DEAE-Sephadex (A-50) or else was concentrated by dialysis against 20% soluble polyvinyl pyrrolidinone (mol wt 40,000; Matheson Coleman and Bell, N. J.) and used for antisera production.

Antigen Preparation from Bacteria. The pellet of tumorigenic (strain 4-32) or nontumorigenic (strain 11 NV6) bacteria obtained by low speed centrifugation was washed three or four times with isotonic saline (0.85% NaCl). The bacterial suspension (about 5 mg/ml) in isotonic saline was next heated at 60 C for 15 min. The heat-killed bacterial preparation was

then sonicated for 5 min at full power in a 20-kc/sec Bronwill Biosonic Sonicator, and the preparation obtained was used for antiserum production.

Protein Extraction from Tumorigenic Bacteria for Chromatography. The bacterial pellet (about 5 g), after being washed twice with medium A, was homogenized with a mortar and pestle with 5 g of acid-washed glass beads (Superbrite Glassbeads P-3909, 3M Company, N. J.) and 25 ml of medium A. The homogenate was centrifuged (30,000*g*, 30 min), and the supernatant obtained was dialyzed against medium A as described earlier for the plant tissue.

Antisera Production. Antisera were produced in white New Zealand rabbits by a series of intramuscular thigh injections of each preparation emulsified in Freund's incomplete adjuvant (Difco Laboratories, Detroit). In the case of the callus and tumor antigens (about 2–2.5 mg of protein per milliliter), 5 or 6 injections were administered at 3-day intervals, but the final injection was given 7 days after the preceding injection. With the bacterial antigen, however, two injections a week apart gave satisfactory antibody production. Bleedings were taken by cardiac puncture about every 7 days. Blood volumes per bleeding were routinely 10 ml.

Serology. Immunodiffusion tests were performed in clear plastic Petri dishes (60 × 15 mm) containing a 4-mm layer of agar in which reactant wells were cut at desired equal distances with a no. 1 corkborer for the peripheral wells and a no. 2 corkborer for the central well. Agar (Nobel agar, Difco, Detroit) was prepared at a concentration of 0.7% with 0.015 M sodium azide and 0.015 M sodium chloride. Reactions were allowed to develop at room temperature and were viewed against a black mat background with oblique lighting.

Absorption tests were done by adding 0.25 ml of a concentrated preparation of the antigen (3.5 mg of protein per milliliter) to 1 ml of antiserum. The tubes were stored at 25 C for 5 hr, and the immunoprecipitate was removed by low speed centrifugation. This procedure was repeated three or four times to ensure complete harvest of cross-reacting antigens. A fraction of the supernatant was tested in immunodiffusion plates after each increment of the antigen. When further absorption did not occur, tests were also conducted at 2 C to enhance reactions between weakly reacting systems (2).

Column Chromatography. A DEAE-Sephadex (A-50) column (2 × 30 cm) was prepared and equilibrated with buffer A as described by Atkin and Srivastava (1). Proteins from the column were eluted by a combined stepwise and linear gradient elution procedure. An initial elution with 300 ml of buffer A was followed by two steps of linear salt gradient obtained first by mixing 300 ml of buffer A with 300 ml of buffer A containing 0.4 M NaCl and then by mixing 200 ml of buffer A containing 0.4 M NaCl with 200 ml of buffer A containing 1 M NaCl. Further elution was done with buffer A containing 1 M NaCl (150) ml), with 0.5 N NaOH (175 ml), with 0.75 N NaOH (150 ml), and finally with 1 N NaOH (200 ml). All steps were performed at 2 C. Sixty-drop (5.2 ml) fractions were collected at a flow rate of 15 to 20 ml/hr. The final elution with 1 N NaOH was carried out at room temperature, and 50-ml fractions were collected. The $A_{280\ nm}$ of each fraction was read against appropriate blanks.

Precipitation Reaction with Column Fractions. Aliquots (0.5 ml) of column fractions 9, 12, and 13 from the tumor tissue and aliquots (0.5 ml) of fractions 10, 14, and 15 from the tumorigenic bacteria were mixed together with 1 ml of antiserum prepared against callus, tumor, and tumorigenic bacteria. The controls consisted of column fractions alone and antisera alone. Formation of precipitate was observed during a standing period of 5 hr at room temperature and then 16 hr at 2 C.

RESULTS AND DISCUSSION

During preliminary serological study, slide agglutination tests were performed as a rapid means for demonstrating qualitative relationships between various antigens and antibodies. Anti-tumor (T) serum gave a positive reaction against homologous antigens and against callus (C) as well as tumorigenic (TB) and nontumorigenic (NTB) bacterial antigens. The instensity of agglutination varied, however. The anti-T serum reacted strongly against TB antigens as compared with NTB antigens. Anti-C serum failed to react against TB and NTB antigens, but gave positive reactions against C and T antigens. Anti-NTB serum reacted strongly against NTB and TB antigens, but gave a very weak reaction against T antigens.

These findings demonstrate that some cross-reacting antigens were present in the T antigen preparation as tested against anti-TB serum. Anti-NTB serum gave a precipitate too, but one that differed considerably in density from that given by anti-TB serum. In subsequent study, immunodiffusion tests were conducted (4). From Figure 1a it is apparent that four precipitin lines were formed (central well) when T antigens were tested against anti-TB serum, and five or six precipitin lines were formed (side wells) when the serum was tested against homologous antigens. TB (side wells) and T antigens, however, when tested against anti-NTB serum, gave only three and two precipitin lines, respectively (Fig. 1b). This difference in the number of precipitin lines indicates that some antigens in tumor tissue are common to TB and NTB whereas others are different.

Anti-TB serum gave no precipitin bands when tested against callus antigens, but gave four, three, and two precipitin bands when tested against TB, NTB, and T antigens respectively (Fig. 1c).

If it is true that bacteria-specific proteins are present in tumor tissue, it should be possible to detect cross-reacting antigens in anti-T serum even when it is absorbed with a callus antigen preparation. Figure 1d demonstrates that anti-T serum, first absorbed with C antigens and then reacted with T, TB, and NTB antigens, gave three distinct bands against T antigens, one distinct band and two faint but specific bands against TB antigens, one distinct band and one faint band against NTB antigens, and absolutely no reaction against callus antigens. These findings strongly support the concept that bacteria-specific proteins are present in tumor tissue.

Next an attempt was made at fractionating the soluble proteins of tumor tissue and callus tissue by DEAE-Sephadex column chromatography. The typical DEAE-Sephadex elution profiles of the proteins from tumor and callus tissue presented in Figure 2 show at least three distinct protein peaks that are present in tumor tissue but which were not detected in callus tissue. All of these three peaks (9, 13, and 14) appeared in the region of the 0–0.4 M sodium chloride gradient, and together amount to 21% of the total $A_{280\ nm}$ units applied to the column. Out of the total $A_{280\ nm}$ units applied, 89.1% for the tumor tissue and 91.6% for the callus tissue were eluted out of the column (Table I).

Besides these qualitative differences, there are distinct quantitative differences between the tumor tissue and the callus tissue proteins (Table I). For example, proteins under peaks number 4, 5, 20, and 21 are significantly greater in quantity in each case in tumor tissue than in callus tissue, whereas proteins under peaks number 6, 8, 10, and 15 for callus tissue are greater in quantity than those under the corresponding peaks for tumor tissue (Table I).

Antigens from peaks number 9, 13, and 14 of the tumor tissue (Fig. 1) were later found to be bacteria specific. Antigens from each of these peaks were allowed to react with anti-

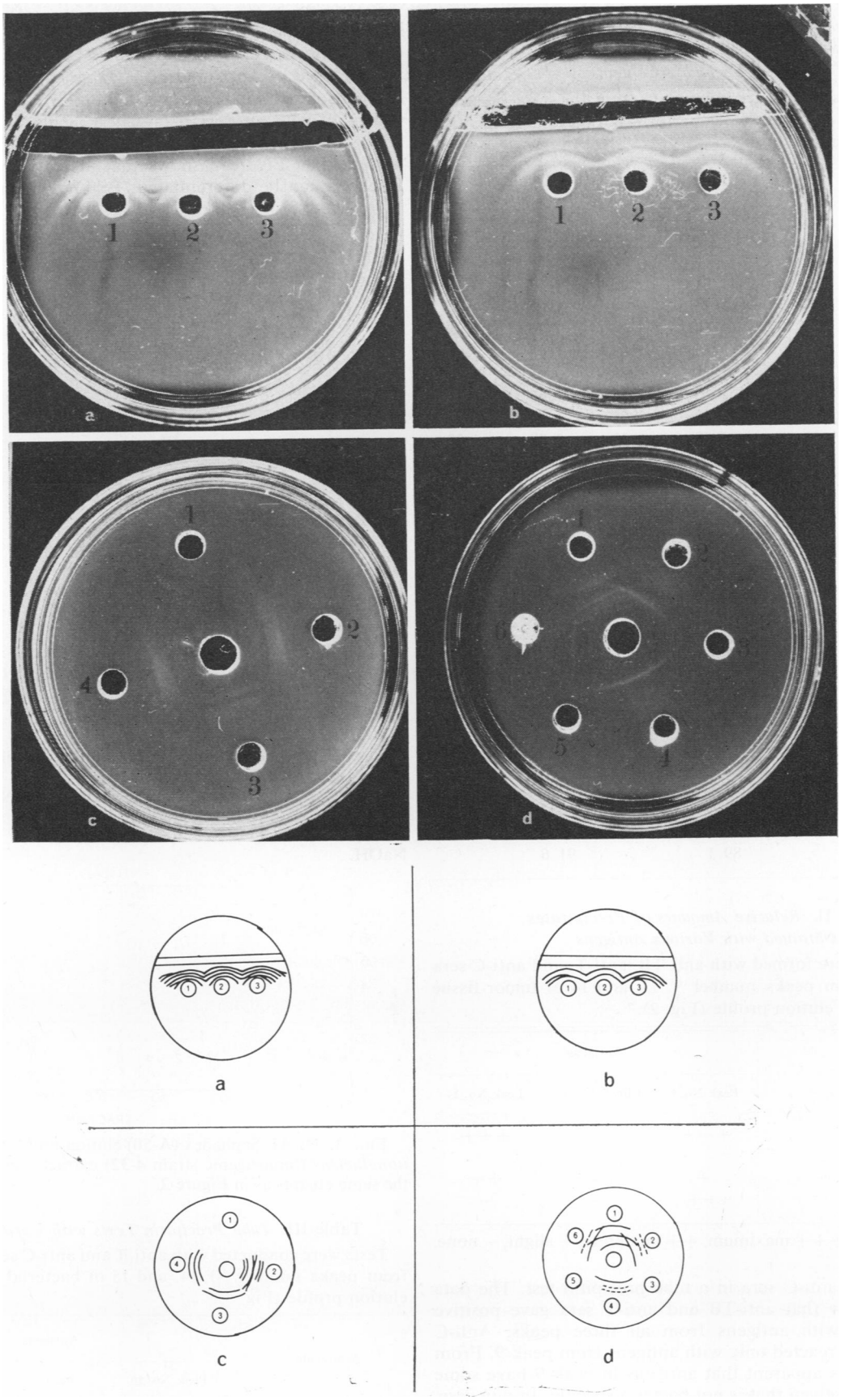

FIG. 1. Photograph and diagrammatic representation of the immunodiffusion patterns of a: TB (wells 1, 3) and T (well 2) antigens against anti-TB serum (trough); b: TB (wells 1, 3) and T (well 2) antigens against anti-NTB serum (trough); c: anti-TB serum (central well) against C (peripheral well 1), TB (well 2), T (well 3), and NTB (well 4) antigens; d: anti-T serum cross-absorbed with C antigens (central well) against T (peripheral well 1), TB (wells 2 and 4), C (wells 3 and 5), and NTB (well 6) antigens.

Table I. *Amounts of Soluble Protein Components of Tumor and Callus Tissue when Separated on a DEAE-Sephadex Column*

Absorbance estimates are calculated from the data presented in Figure 2. The experiment was performed 4 times, and identical results were obtained.

Peak No.	Soluble Protein Components			
	Tumor	Relative %	Callus	Relative %
	A_{280} nm ml/g tissue			
1	1.61	2.20	1.49	3.10
2	1.31	1.79	1.24	2.58
3	3.98	5.44	3.75	7.80
4	3.68	5.03	1.48	3.08
5	1.98	2.70	0.91	1.89
6	1.10	1.50	1.66	3.45
7	2.89	3.95	1.67	3.47
8	0.98	1.33	2.08	4.32
9	7.07	9.66	...	...
10	1.93	2.64	2.87	5.97
11	2.89	3.95	3.44	7.16
12	3.21	4.38	2.58	5.37
13	2.43	3.32	...	...
14	5.99	8.18	...	...
15	4.51	6.16	7.56	15.73
16	1.73	2.36	1.21	2.51
17	1.99	2.72	1.36	2.83
18	3.67	5.01	3.45	7.18
19	7.75	10.59	5.29	11.01
20	8.17	11.16	3.04	6.32
21	4.10	5.60	1.08	2.24
Total $A_{280\ nm}$ units applied to column	82.50		52.5	
$A_{280\ nm}$ units recovered	73.15		48.04	
Recovery (%)	89.1		91.6	

Table II. *Relative Amounts of Precipitates Obtained with Various Antigens*

Precipitates were formed with anti-TB, anti-T, and anti-C sera and antigens from peaks number 9, 13, and 14 of tumor-tissue DEAE-Sephadex elution profile (Fig. 2).

Antiserum	Antigens		
	Peak No. 9	Peak No. 13	Peak No. 14
A. tumefaciens (tumorigenic)	+++[1]	++	+++
Tumor	+	+++	++
Callus	++	−	−

[1] Precipitates: +++ maximum, ++ moderate, + slight, − none.

TB, anti-T, and anti-C sera in a tube precipitin test. The data in Table II show that anti-TB and anti-T sera gave positive precipitin tests with antigens from all three peaks. Anti-C serum, however, reacted only with antigens from peak 9. From this reaction, it is apparent that antigens in peak 9 have some contaminating antigen that is not bacteria specific. In any case, antigens in peaks number 13 and 14 are exclusively bacteria specific.

The results of this study thus show that bacteria-specific proteins are present in tumor tissue, as is evident from serological data, and that these proteins can be separated by column chromatography. These observations led us to examine the bacterial proteins under the same conditions to see if any of the bacterial antigens separated on DEAE-Sephadex column will react with antiserum prepared against tumor tissue. The typical DEAE-Sephadex elution profile of bacterial proteins presented in Figure 3 shows that at least 26 distinct protein fractions can be differentiated. Peaks number 10, 14, and 15

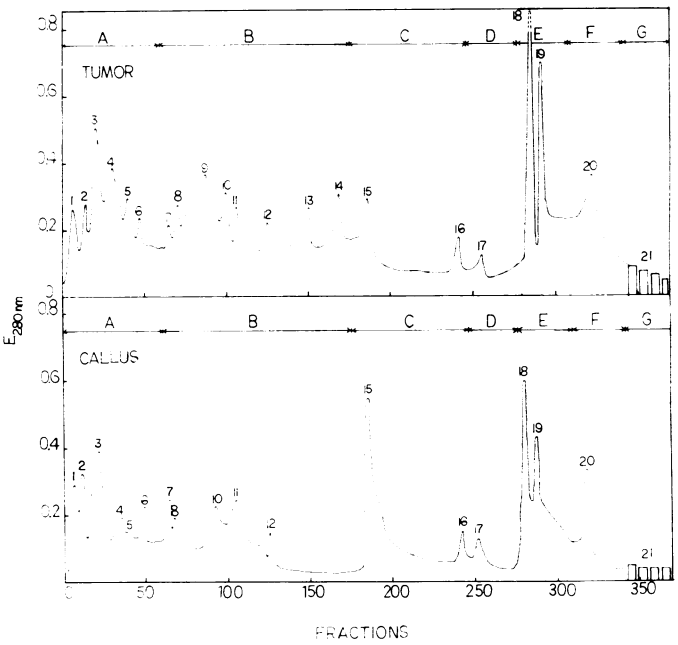

FIG. 2. DEAE-Sephadex (A-50) elution profiles of tumor and callus tissue extracts. A: 50 mM tris-HCl, pH 7.4, containing 5 mM cysteine; B: a linear gradient of 0–0.4 M NaCl in eluant A; C: 0.4–1.0 M NaCl linear gradient in eluant A; D: eluant A containing 1 M NaCl; E: 0.5 N NaOH; F: 0.75 N NaOH; G: 1.0 N NaOH.

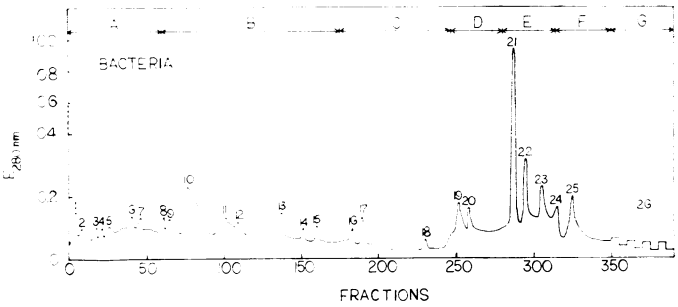

FIG. 3. DEAE-Sephadex (A-50) elution profile of *Agrobacterium tumefaciens* (tumorigenic strain 4-32) extract. Letters A–G refer to the same eluants as in Figure 2.

Table III. *Tube Precipitin Tests with Various Antigens*

Tests were conducted with anti-T and anti-C serum and antigens from peaks number 10, 14, and 15 of bacterial DEAE-Sephadex elution profile (Fig. 3).

Antiserum	Antigens		
	Peak No. 10	Peak No. 14	Peak No. 15
Tumor	+++[1]	+	++
Callus	−	−	−

[1] Precipitates: +++ maximum, ++ moderate, + slight, − none.

of the bacterial DEAE-Sephadex elution profile (Fig. 3) roughly correspond to peaks number 9, 13, and 14, respectively, of the tumor-tissue DEAE-Sephadex elution profile (Fig. 2). Antigens from these three peaks of the bacterial profile, when allowed to react separately with anti-T serum, gave a positive precipitin test in each case. Peak number 10 gave very strong reactions, whereas peaks number 14 and 15 gave slight and moderate reactions. None of these three peaks gave any reaction when tested against C antiserum (Table III).

There are several reports in the current literature that indirectly support our findings. Quetier *et al.* (7) have found complementarity between *A. tumefaciens* DNA and crown gall tumor-tissue DNA. Milo and Srivastava (6) have detected RNA in crown gall tissue complementary to *A. tumefaciens* DNA. While the present study was in progress, Schilperoort *et al.* (9) published a preliminary communication reporting the presence, in sterile crown gall tumors, of antigens cross-reacting with *A. tumefaciens*.

In the present study, however, we have obtained direct evidence of the presence of bacteria-specific proteins in bacteria-free crown gall tumor tissue, and we have found that these proteins can be fractionated by column chromatography, and also that they show serological cross reactivity to one another. These results fully support the concept that bacteria-free tumor tissue contains the integrated *A. tumefaciens* genome and that integration of the bacterial genome with the host genome confers upon the host cells the properties associated with the tumor tissue. Research dealing with the nature of these bacteria-specific proteins detected in bacteria-free tumor tissue extracts and the biological significance of these proteins is in progress.

LITERATURE CITED

1. Atkin, R. K. and B. I. Srivastava. 1970. Studies on protein synthesis by senescing and kinetin-treated barley leaves. Physiol. Plant. 23: 304–315.
2. Boyd, W. C. 1956. Fundamentals of Immunology, 3rd Ed. Interscience Publishers, Inc., New York.
3. Bruan, A. C. 1962. Tumor inception and development in the crown gall disease. Annu. Rev. Plant. Physiol. 13: 533–558.
4. Crowle, A. J. 1958. A simplified micro double-diffusion agar precipitin technique. J. Lab. Clin. Med. 52: 784–787.
5. McCown, B. H., G. E. Beck, and T. C. Hall. 1968. Plant leaf and stem proteins. I. Extraction and electrophoretic separation of the basic water soluble fraction. Plant Physiol. 43: 578–582.
6. Milo, G. E., and B. I. Srivastava. 1969. RNA-DNA hybridization studies with the crown gall bacteria and the tobacco tumor tissue. Biochem. Biophys. Res. Commun. 34: 196–199.
7. Quetier, F., T. Huguet, and E. Guille. 1969. Induction of crown gall: Partial homology between tumor-cell DNA, bacterial DNA and the G + C rich DNA of stressed normal cells. Biochem. Biophys. Res. Commun. 34: 128–133.
8. Reddi, K. K. 1966. Ribonuclease induction in cells transformed by *Agrobacterium tumefaciens*. Proc. Nat. Acad. Sci. U. S. A. 56: 1204–1214.
9. Schilperoort, R. A., W. H. Meijs, G. M. Pippel, and H. Veldstra. 1969. *Agrobacterium tumefaciens* cross-reacting antigens in sterile crown-gall tumors. FEBS Lett. 3: 173–176.
10. Srivastava, B. I. S. 1967. Effect of kinetin on biochemical changes in excised barley leaves in tobacco pith tissue culture. Ann. N. Y. Acad. Sci. 144: 260–278.
11. Srivastava, B. I. S. 1968. Patterns of nucleic acid synthesis in normal and crown gall tumor tissue cultures of tobacco. Arch. Biochem. Biophys. 125: 817–823.
12. Srivastava, B. I. S. 1971. Studies on chromatin from normal and crown gall tumor tissue cultures of tobacco. Physiol. Plant Path. In press.
13. Srivastava, B. I. S. 1965. Studies on nucleic acids from healthy and tumorous tomato stem tissue. Biochim. Biophys. Acta 103: 349–352.
14. Srivastava, B. I. S. and K. C. Chadha. 1970. Liberation of *Agrobacterium tumefaciens* DNA from the crown gall tumor cell DNA by shearing. Biochem. Biophys. Res. Commun. 40: 968–972.