presented.

1. Williams, W. L., Ellenbogen, L., and Esposito, R. G., PROC. SOC. EXP. BIOL. AND MED., 1954, v87, 400.

2. Hastings, A. B., Teng, C. T., Nesbett, F. B., and Sinex, F. M., *J. Biol. Chem.*, 1952, v194, 69.

3. Korkes, S., Del Campillo, A., Gunsalus, I. C., and Ochoa, S., *ibid.*, 1951, v193, 733.

4. Wijmenga, H. G., Thompson, K. W., Stern, K. G., and O'Connell, D. J., *Biochem. et Biophys. Acta*, 1954, v13, 144.

5. Miller, O. N., *Bull. Tulane U. Med. Fac.*, 1957, v16, 115.

6. Bird, O. D., and Hoevet, B., *J. Biol. Chem.*, 1951, v190, 181.

7. Miller, O. N., *Arch. Biochem. and Biophys.*, 1957.

Received June 11, 1957. P.S.E.B.M., 1957, v96.

Plasma Protein Changes in Parakeets with Pituitary Tumors.* (23389)

HANS G. SCHLUMBERGER† AND ROBERT L. WALL

Departments of Pathology and Medicine, College of Medicine, Ohio State University, Columbus

Among the commonest tumors of the parakeet *Melopsittacus undulatus,* are adenomas and carcinomas of the pituitary(1). Over 160 cases have been examined in this laboratory, and in the past 4 years one of the tumors has been carried through 14 passages by subcutaneous transplantation(2). Both primary tumors and transplants often produce marked obesity and hyperglycemia. Bioassays of the transplanted tumors suggest the presence of somatotropin, but they have been negative for other pituitary hormones(2). The plasma proteins show a striking quantitative change in the electrophoretic pattern (Fig. 1), and a qualitative difference from the normal after ultracentrifugation.

Materials and methods. Tumors to be transplanted were minced in 0.9% NaCl and injected beneath the skin of the breast. Cortisone, total body x-irradiation, or other form of pretreatment of recipient birds was never used. Total plasma protein and blood sugar were determined by a microphotometric application of the Folin-Ciocalteu procedure(3) and by the Folin-Wu technic(4) respectively. Parakeets with primary pituitary tumors were exsanguinated by cardiac puncture. Clotting was prevented with heparin in most instances; in several cases no heparin was used and the electrophoretic pattern of the serum so obtained compared with that of plasma. The method of paper electrophoresis did not demonstrate a significant difference between serum and plasma. No separate peak corresponding to fibrinogen could be detected in the plasma, a finding similar to that observed in mice(5) and fowls(6). Birds with transplanted pituitary tumors were also exsanguinated, particularly if blood sugar determinations and ultracentrifugation were to be carried out on the same sample. When serial protein determinations were made on a single bird, blood was collected in heparinized capillary tubes from a wing vein. Plasma was applied in amounts of 10 cu mm to Whatman 3-mm filter paper that measured 10 x 14 cm, providing space for simultaneous testing of several samples. Using a conventional vertical principle electrophoretic cell designed by one of us (R.W.), Veronal buffer at pH 8.6, u 0.05 with 5% glycerine, a current of 150-170 volts delivering 15-20 milliamperes was applied to the paper for 4 hours. After heat fixation the papers were stained with Bromphenol blue for protein and Sudan black B for lipoprotein. The papers were then cut into strips each bearing the pattern of a single sample, and scanned with a Spinco Analytrol that permitted simultaneous area integration. Pooled human serum served as a control and mobility reference. The protein fractions in parakeet sera were not isolated, therefore their dye-binding capacities could not be de-

*Supported by grants from Nat. Cancer Inst., USPHS and Am. Cancer Soc.

† Present address, Medical Center, University of Arkansas, Little Rock.

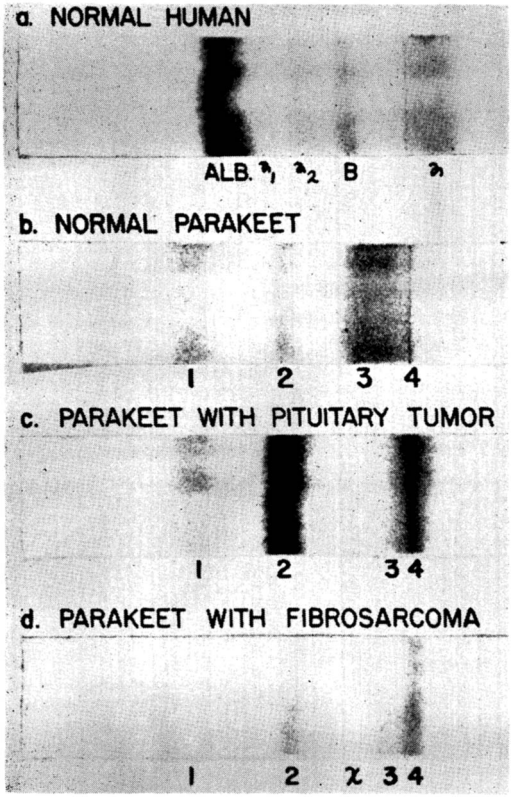

FIG. 1. Representative electrophoretic patterns of parakeet plasma with a human serum pattern as mobility reference.

termined directly. They were assumed to be similar to those of the identically treated human sera. This assumption is supported by the results of boundary electrophoresis, which were similar to those obtained by paper electrophoresis. An Aminco-Stern boundary electrophoretic apparatus was used to determine the electrophoretic mobilities of plasma protein components with Veronal buffer, pH 8.6, u 0.1. To supply the relatively large volume of plasma needed for these studies it was necessary to use pooled samples from several birds. Mobilities were calculated from the ascending boundaries and are reported in Tiselius units. Analysis by ultracentrifugation was performed on a Spinco Model E optical ultracentrifuge at 59,780 r.p.m. Plasma from individual birds as well as samples of pooled plasma from several birds were used. Sedimentation constants were calculated from 3 or more dilutions of each sample and are reported in Svedberg units.

Results. Normal Parakeets. In 15 normal birds the average total plasma protein was 3.34 g %, range 1.98 to 4.74 g %. The plasma from each of 29 normal parakeets and the pooled plasma of 40 normal birds was subjected to zone and boundary electrophoresis. The birds ranged in age from 2 months to 2 years, the sexes were equally distributed. Four major components are recognizable(7), on the basis of their mobilities they are similar to those found in other birds(8,9) and man. Component 1 has a mobility of 8.5, and may be albumin. The components 2, 3, and 4 have mobilities of 4.2, 3.25, and 2.25 respectively and are probably globulins; however, they have not yet been identified by chemical methods. There is considerable individual variation in the quantity of each of these 4 fractions present in the plasma (Fig. 2). The average percentile of component 1 is 32 (range 17-36%), of 2 is 16 (6-28%), of 3 is 22 (8-45%), and of 4 is 29 (17-48%). An affinity for the lipid stain Sudan black B was pronounced in component 1, less intense in 3 and 4 which stained as a unit, and barely perceptible in component 2.

Birds with primary pituitary tumors. In 9 parakeets a diagnosis of pituitary tumor could be made before death. The birds were exsanguinated by cardiac puncture and the presence of a pituitary tumor confirmed at autopsy (Table I). The plasma electrophoretic pattern was characterized by a striking increase in component 2 (Fig. 3) with an average percentile of 57 (range 34-67%) as compared with a normal of 16 (range 6-28%). The other components were correspondingly decreased, particularly component 1 which fell to 10% (range 3-16); component 3 averaged 14% (range 5-34), and component 4 was 21% (3-35). Several of the birds were

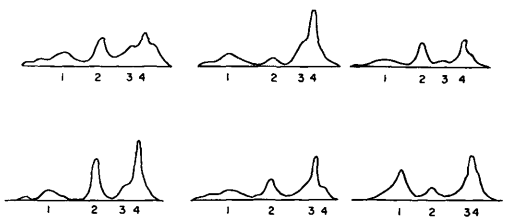

FIG. 2. Plasma protein patterns of 6 normal parakeets showing usual quantitative variation in the 4 components.

TABLE I. Birds with Primary Pituitary Tumors.

Date	Sex	Age (yr)	% tot. prot. as component 2*	Remarks
3/ 3/55	♂	4	56	Fatty and glycogen-rich liver, blood sugar 366 mg %
18	♀		50	5 mm tumor, blood sugar 97 mg %
4/13	♂	1	64	Tumor infiltrates 4th ventricle, fatty liver
10/13	♀		53.5	Large tumor invading retro-orbital tissue
3/16/56	♂		57.4	2 mm tumor, glycogen-rich liver
12/ 6	♀	2	67	Large tumor invading base of skull
1/29/57	♀	2	61	4 mm invasive tumor, glycogen-rich liver
2/ 6	♂	4	66	5 mm infiltrating tumor, fatty liver
3/ 7	♂	3	34	Large tumor invading skull and retro-orbital tissue

* In 29 normal parakeets avg value of component 2 was 16% (range 6-28%).

obese, in one the blood sugar level was elevated to 366 mg%; the normal is 150-250 mg%. Except for frequency of fatty and glycogen-rich liver, no consistent changes were observed in gross or microscopic appearance of the viscera or other endocrine glands.

Birds with transplanted pituitary tumors. On June 18, 1953 a massive pituitary tumor was found invading the roof of the mouth and retro-orbital tissue of a male parakeet. The tumor grow well when transplanted subcutaneously in other parakeets and since then has been carried through 14 serial passages. Electrophoretic studies were made on 23 birds bearing the tumor in its 3rd to 9th, and 12th passages (Table II). At the time of bleeding all but 5 transplants were over 100 days old; the average was 127 days with a range of 38-240 days. Only one tumor was less than 1 cm in diameter; the average was 2.1 cm with a range of 0.5 to 4.5 cm. Blood sugar levels were obtained in 13 instances on the same blood sample that was used for electrophoresis. Three were high normal; *i.e.*, 171, 212, 250 mg%, the remaining 9 were elevated ranging from 324 to 1768 mg% with an average of 887 mg%. The changes produced in the plasma electrophoretic pattern were identical to those observed in plasma of birds with a primary pituitary tumor; *viz.*, a pronounced increase in component 2 (Fig. 3). The average for component 2 in these birds was 51.5% of the total protein, with a range of 33-70%. This increase was very consistent and not altered by the number of passages, size of tumor, its duration of growth, or the blood sugar level. Nor was the increase of component 2 reflected in the total protein which when determined in 9 birds with a tumor in the 6th or 8th passage ranged from 2.64 to 5.93 g %. The average of 4.23 g % was slightly lower than the normal average of 4.74 g %.

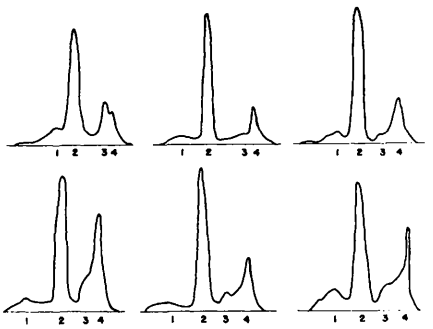

FIG. 3. Plasma protein patterns of 6 parakeets with pituitary tumors showing characteristic increase of component 2. Top row, birds with primary pituitary tumors; bottom row, birds with transplanted pituitary tumors.

To determine the time when the protein changes first become apparent, parakeets were inoculated subcutaneously with tumor from the 7th or 8th passages. One male and one female were then exsanguinated on each day for the first 7 days and then at weekly intervals for 14 weeks. Plasma electrophoresis first showed a definite increase of component 2 on the third day after transplantation, by the sixth day 45 to 65% of the total protein was present as component 2, a level 4 times that of the average normal. At this time the transplants were still very small and there was no gross evidence of growth. Blood sugar determinations made on the same blood sample as that used for electrophoresis, showed

TABLE II. Birds with Transplanted Pituitary Tumors.

Passage	Sex	Age of transplant (days)	Size of transplant (cm)	Blood sugar (mg %)	% tot. prot. as component 2
2	♀	180	1.5	1408	47
2	♂	240	1.0	1076	58
3	♀	137	3.5	1768	45
3	♀	150	2.0	504	60
3	♀	180	1.5	1332	60
3	♂	212	3.5	171	65
4	♂	78	1.5	212	59
4	♂	95	2.0	708	64
4	♀	125	1.5	250	35
4	♀	175	3.0	468	45
5	♀	115	2.0		55
5	♀	122	3.0	759	59
6	♂	80	1.5		56
6	♂	86	1.0		70
6	♂	93	1.5		47
7	♂	66	2.0		45
7	♂	185	3.0		41
8	♂	38	.5		33
9	♀	137	3.0		44
9	♂	137	2.0		55
12	♀	106	3.0	324	51
12	♂	106	4.5	352	44

considerable variability, in contrast to the constancy of the plasma protein changes. The first elevated blood sugar (298 mg%) was found in a male bird 9 weeks after transplantation when the tumor measured 8 mm in diameter. In one female with a 1.5 mm transplant, the blood sugar was 789 mg% by the tenth week and in a male with a 2 cm tumor transplant at 12 weeks, the blood sugar was 1220 mg%. However, at this time some birds with tumors of equal size still had blood sugar levels in the normal range.

Changes in plasma proteins following excision of the tumor were determined in 12 parakeets bearing 8-week-old transplants. In all birds component 2 of the plasma was elevated at the time of operation. 2 weeks later it had fallen to normal in all but 2 birds. In these, removal of the transplant had been incomplete as was indicated by a recurrence of the tumor at the original site within the following 2 months. During this time the plasma electrophoretic pattern of the other 10 birds remained normal.

Birds with other primary tumors. Electrophoresis was carried out on plasma of 5 birds with primary spontaneous tumors in which the pituitary was normal. Histologically 4 were fibrosarcomas, one was a rhabdomyosarcoma. In none did the electrophoretic pattern show an abnormal increase in component 2; instead, there was a decrease of all fractions associated with a marked hypoproteinemia (Table III, Fig. 1).

Birds with other transplanted tumors. In this laboratory 3 parakeet fibrosarcomas are being carried as subcutaneous transplants (10). The tumors originated in the retroperitoneal, subcutaneous, and retro-orbital tissues respectively. Electrophoretic patterns were obtained on the plasma of 16 birds bearing transplants of these tumors that were in the second to twelfth passage and measured 1.5 to 3.5 cm in diameter. The percentile distribution of the 4 components was within the normal range except in one bird where component 2 was greatly increased. At autopsy this bird proved to have a primary pituitary tumor.

A transplantable methylcholanthrene-induced skin carcinoma of the parakeet(11) provided an epithelial tumor to compare with the pituitary neoplasm for its effect on the plasma proteins. Birds bearing these trans-

TABLE III. Birds with Other Primary Tumors.

Sex	Age (yr)	Tumor Type	Tumor Size	Tumor Site	% tot. prot. as component 2	Remarks
♀	2	Fibrosarcoma	2.5 × 2.5	Wing	14	Grew on transplantation
♀	2	"	2 × 1.5	Thigh	5	Met. to lung, kidney, liver
♂	3	"	2.5 × 2	Orbit	20	Met. of sarcoma to spleen and liver
		Adenocarcinoma	1.5 × 1	Kidney		
♂	–	Fibrosarcoma	2.3 × 2	Thigh	30*	No growth of transplants
♀	–	Rhabdomyosarcoma	2 × 2	Neck	12	*Idem*

* Marked hypoproteinemia, extremely low values for all components.

planted skin carcinomas in the second and third passage were exsanguinated when the tumors had reached a diameter of 2 to 3.5 cm. The electrophoretic pattern of the plasma protein was in the normal range and did not show the increase in component 2 characteristic of pituitary tumors.

Ultracentrifugation of plasma proteins. Four ultracentrifuge analyses were made on 2 different collections of pooled plasma from normal parakeets. Three components were seen: 1) a high density fraction with sedimentation constant of 19.7 representing approximately 5-8% of total protein, 2) a slower component that comprised approximately 5% of the protein and had a constant of 6.2, 3) the remaining 85-90% of the plasma protein with a sedimentation constant of 3.6. Nine analyses by ultracentrifugation were made on 2 different collections of pooled plasma from parakeets bearing transplanted pituitary tumors. The samples were first examined by electrophoresis for the presence of the abnormal component 2, the latter was found to represent 52% of the total protein. With ultracentrifugation 4 fractions were identified: 1) a high density component with a sedimentation constant of 17.9 representing approximately 5% of the protein, 2) a slower fraction having a sedimentation constant of 6.5, comprising 3-5% of the protein, 3) a new component with a sedimentation constant of 5.2 that constituted about 35% of the protein, 4) a fraction with a constant of 4.0 that contained the remaining 50-55% of protein. A fifth component showing flotation characteristics at the specific gravity of the diluent suggestive of a lipoprotein was also present.

The third component found in the ultracentrifuged plasma of tumor bearing birds is not seen in the plasma of normal parakeets. It may be a protein that is present in normal plasma in such small amounts that it is not detected by this method, or it is a new protein related to the presence of the pituitary tumor transplants.

Discussion. On the basis of its mobility during electrophoresis, the plasma protein fraction that is increased in parakeets with pituitary tumors is tentatively identified as a globulin. In man, serum globulins are increased in a wide variety of conditions but there is no consistent change characteristic of neoplastic disease(12). However, in multiple myeloma very large amounts of beta or gamma globulin may be demonstrated by electrophoresis(13). Reports on serum proteins of tumor-bearing rats and mice indicate great variability. Recently Bernfield and Homburger(5) have described a substantial increase in plasma alpha globulin of mice during 12 days after implantation of Sarcoma 180; upon further growth of the tumor the alpha globulin again decreases. Studies by Potter *et al.*(14) have demonstrated an increase of gamma globulin in a transmissible neoplasm of mice that closely resembles human multiple myeloma. These investigators suggest that the abnormal protein is produced by the neoplastic cells, a view supported by direct quantitative relationship between size of tumor and the amount of abnormal serum component. In the parakeet, component 2 is already markedly increased

by the 3rd to 4th day after transplantation of 2-3 cu mm of tumor and before there is any visible evidence of growth. Although component 2 often shows a 5-fold increase, no consistent change in total protein was found. Preliminary studies on saline extracts of the tumor have indicated the presence of component 2, but in lower concentration than in the blood of the host birds.

It is possible that the protein is formed by a target organ, *e.g.* the reticulo-endothelial system, which is activated by a hormone produced by the pituitary tumor. A hormone, perhaps somatotropin(2), secreted by the pituitary tumor probably produces the hyperglycemia that is often observed to accompany changes in plasma protein. The role of a hormone in altering serum proteins was recently reported by Schjeide and Urist(15). They observed that in roosters 24 hours after intramuscular injection of estrone, there is a large increase in serum beta lipoproteins, nearly complete disappearance of alpha lipoproteins and the appearance of 2 new components—a phosphoprotein and a lipoprotein. These changes reach a maximum in 5 days. The time elapsing between injections of estrogen and the appearance of new serum protein components in the rooster is the same as that observed in the parakeet following transplantation of the pituitary tumor.

The chemical identity of component 2 in the electrophoretic pattern has not yet been determined. The finding of a fourth plasma fraction by ultracentrifugation, which is not present in normal plasma, suggests that most of the increase in component 2 may be due to the production of a qualitatively different protein.

Summary. The first recognized manifestation of a primary or transplanted pituitary tumor in the parakeet is a marked increase in a plasma protein component, tentatively identified as a globulin. Analysis by optical ultracentrifugation suggests that this protein is qualitatively different from any present in normal plasma. Several weeks after appearance of the abnormal protein there is often an associated hyperglycemia and obesity. It is suggested that all these changes express the response of target tissues to one or several hormones secreted by the tumor.

1. Schlumberger, H. G., *Canc. Res.*, 1954, v14, 237.
2. ———, *ibid.*, 1956, v16, 149.
3. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, 1927, v73, 627.
4. Folin, O., and Wu, H., *ibid.*, 1920, v41, 367.
5. Bernfield, P., and Homburger, F., *Canc. Res.*, 1955, v15, 359.
6. Deutsch, H. F., and Goodloe, M. B., *J. Biol. Chem.*, 1945, v161, 1.
7. Wall, R. L., and Schlumberger, H. G., *Science*, 1957, v125, 993.
8. Common, R. H., McKinley, W. P., and Maw, W. A., *ibid.*, 1953, v118, 86.
9. Spector, W. S., *Editor of Handbook of Biological Data*, p25, Table 18, W. B. Saunders Co., Philadelphia, 1956.
10. Schlumberger, H. G., *Fed. Proc.*, 1957, v16, 371.
11. ———, *Canc. Res.*, 1956, v16, 1043.
12. Winzler, R., *Advances in Cancer Research*, p503, Ed. by J. P. Greenstein and A. Haddow, Academic Press, N. Y., 1953.
13. Adams, W. S., Alling, E. L., and Lawrence, J. S., *Am. J. Med.*, 1949, v6, 141.
14. Potter, M., Fahey, J. L., and Pilgrim, H. I., PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 327.
15. Schjeide, O. A., and Urist, M. R., *Science*, 1956, v124, 1242.

Received June 12, 1957. P.S.E.B.M., 1957, v96.