A STUDY OF MONOZYGOUS QUADRUPLET ARMADILLOS IN RELATION TO MAMMALIAN INHERITANCE

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In 1951, studies in our laboratories revealed, for example, that among Wistar rats that had been brother-sister mated for 101 generations and were consuming exactly the same diet, individual urinary excretion patterns showed wide diversity. As an instance, in a small group of these animals, urinary phosphate excretion varied 11-fold and lysine excretion at least 14-fold.[1]

Other investigations have shown that within groups of closely inbred animals (including fowl), enormous variations—up to 60-fold or more—may readily be observed with respect to such items as the voluntary consumption of alcohol, choices of various types of foodstuffs, and tendency to exercise.[2] We have recently been concerned also with the wide differences in the nutritional needs of individual inbred animals for vitamins A and C.[3, 4] All these wide disparities call for more adequate explanation.

We have wondered whether unknown factors aside from the gene pool itself do not control the intricate process of differentiation, *particularly the extent to which each of the numerous types of differentiated cells proliferates.* If there is this sort of control, even animals with identical complements of genes could have organs that differ in size, and every hormone could be produced in characteristic amounts in each individual animal, depending upon the extent to which the various hormone-producing cells had proliferated during development. Hormone patterns are of particular interest because we have some reason to think that hormonal differences are related to several of the areas in which we observe wide differences (food choices, etc.).

Clearly it would be difficult to test this interpretation as long as inbred animals with only similar gene pools are studied, because disparities which are observed might always be attributed to slight differences in the gene complements.

In order to find a more clear-cut answer to the question, we have studied a number of structural and biochemical parameters in newborn quadruplet armadillos. The reproduction of the nine-banded armadillo has been thoroughly studied by Newman and Patterson,[5-7] who found the normal mode to involve the regular production of monozygous quadruplets. Sets of animals are thus produced which should have, barring rare mutations, exactly the same genes. It is interesting that in early work, Newman and Patterson commented on variations in scute numbers and aberrations in banding. These differences, though small, may be highly significant.

In the process of producing monozygous quadruplets, the single fertilized ovum develops to the blastocyst stage, later becomes implanted, and four primordial buds are formed in two stages. These develop independently. While the chromosomal material of the zygote is duplicated in these buds, it seems un-

likely that the cytoplasmic contents are identical. In view of the complex structure of a fertilized ovum, which contains numerous structural features and organelles arranged asymmetrically, it would be impossible for such a cell to divide into two halves, each cytoplasmically equivalent to the other.

If then the factors which control the extent of the proliferation of the various differentiated cells reside in the cytoplasm (or outside the chromosomes), we should expect that among monozygous quadruplet armadillos, there would be varying differences in the sizes of organs and in biochemical parameters related to these varying organ sizes. If this postulate is valid, the individual armadillos of a quadruplet set should show small differences only if each of the four primordial buds had accidentally incorporated approximately the same amount of effective extrachromosomal agents.

Materials and Methods.—Sixty-two adult female armadillos were obtained from a commercial supplier, numbered 1–62, and cared for as described in more detail elsewhere.[8] They were kept under constant surveillance during March and April so that the young could be collected as soon as born. Even so, there was evidence that some young were eaten or otherwise injured at birth; to circumvent this, six quadruplet sets were delivered by cesarean section. Only complete quadruplet sets, of which 16 were collected, were included in this study. These sets were given the numbers 3, 7, 14, 15, 21, etc., which had been assigned to the female armadillos which gave birth to them. The individual animals were designated 3A, 3B, 3C, 7A, 7B, etc. One of the sets included an extra animal and therefore constituted a set of quintuplets. This has been observed before.

The newborn were sacrificed immediately after birth (or the cesarean operation) by immersing the head in liquid nitrogen for 1 min. They were then decapitated and the bodies processed in accordance with a careful time schedule as described elsewhere.[8] Organ weights were determined by weighing the organ in question on a Mettler semimicrobalance exactly $1^1/_2$ min after removal of the organ from the freezer.

Fluorometric analyses for epinephrine and norepinephrine were made on the hearts, spleens, adrenals, and brains of all the young, essentially by the method of Shore and Olin.[8, 9] Serotonin analyses were made fluorometrically by the method of Bogdanski *et al.*[8, 10] Serotonin was not found in any heart, spleen, adrenals, or brain, although the method was sensitive and reproducible with as little as 0.01 μg/ml being detectable.

The free amino acids in brain homogenates, after precipitation of the proteins with sulfosalicylic acid, were determined on a relative basis by methods described in more detail elsewhere;[8] chromobeads, Type B resin, and a Technicon autoanalyzer were used. All the methods of analysis were subjected to tests for reproducibility;[8] in every case the reproducibility was far above that required to study differences of the magnitude observed.

The principal results of this study, involving 20 parameters, are summarized in Table 1. More detailed information is presented in Table 2 with respect to one of the quadruplet sets (no. 54) for reasons which will become apparent in the *Discussion*.

Discussion.—Consideration of Table 1 reveals that the differences within quadruplet sets may be very large. For all the 20 parameters listed, the median of the maximum differences observed in the 16 sets is 2.4-fold. It is also clear that the differences may be relatively small. The median of the minimum differences observed in the same sets is 6.5 per cent.

Differences are always present; these were easily measurable within each set of quadruplets for each of the 20 parameters, except that in five sets the norepinephrine in the spleen was present at such low levels that it could not be quantitated.

The factors accounting for these differences are unknown. While it cannot be

TABLE 1. *Summary of principal results.*

	Extreme variations (65 animals)*	Relative differences†	Minimum variation within a set	Relative differences†	Set showing minimum	Maximum variation within a set	Relative differences†	Set showing maximum
1. Body wt	28.6–111.4	4-fold	56.69–58.10	2.5%	7	28.6‡–69.10	2.5-fold	29
2. Scute no.	505–569	12%	536–541	1.%	34	535.–557.	4.%	57
3. Adrenal wt/100 gm	0.202–0.805	4-fold	0.213–0.238	12.%	59	0.405–0.805	99.%	29
4. Brain wt/100 gm	3.203–7.568	2.4-fold	3.972–4.043	1.8%	37	4.629–7.568	63.%	29
5. Heart wt/100 gm	0.388–1.021	2.9-fold	0.425–0.448	5.4%	50	0.527–1.011	92.%	14
6. Kidney wt/100 gm	0.933–1.994	2.1-fold	1.068–1.079	1.%	7	0.943–1.588	68.%	54
7. Liver wt/100 gm	2.114–5.440	2.6-fold	2.114–2.248	6.2%	3	2.950–4.384	49.%	30
8. Spleen wt/100 gm	0.126–0.641	5-fold	0.250–0.282	13.%	61	0.146–0.302	2.1-fold	29
9. Small inst. length, cm	107.–250.	2.3-fold	121.–125.	3.3%	30	148.–250.	70.%	29
10. Norepinephrine, µg in spleen	<0.01–0.130	More than 13-fold			7, 34, 50, 54, 57	<0.01–0.07	More than 7-fold	29
11. Norepinephrine, µg/gm brain	0.020–0.192	86-fold	0.114–0.144	26.%	34	0.024–0.159	6.6-fold	7
12. Norepinephrine, µg/gm adrenals	<0.01–12.12	1200-fold	7.38–7.81	6.%	59	0.035–5.81	16.6-fold	58
13. Epinephrine, µg/gm adrenals	0.05–14.29	286-fold	7.27–8.71	20.%	58	0.05–1.60	32.-fold	54
14. Catecholamine, % present as norepinephrine in adrenals	<0.2–98	490-fold	63.–69	10.%	57	<0.2–28.	140.-fold	34
15. Aspartate in brain homogenate (relative levels)[7]	1.2–10.9	9-fold	1.92–2.74	43.%	29	1.17–6.49	5.5-fold	54
16. Glutamate in brain	9.7–31.1	3-fold	17.39–18.80	8.%	29	15.96–28.27	77%	50
17. Glycine in brain	2.7–15.0	5.6-fold	4.53–4.97	9.%	3	3.72–12.72	3.4-fold	40
18. Alanine in brain	2.1–16.2	7.7-fold	4.64–5.36	16.%	7	3.33–12.29	3.7-fold	54
19. GABA in brain	2.7–17.5	6.5-fold	9.52–9.87	3.6%	37	4.60–10.47	2.3-fold	50
20. Taurine in brain	9.0–29.1	3.2-fold	13.23–14.18	7.%	21	5.54–14.98	2.7-fold	58
	Median 4.5-fold		Median 6.5%				Median 2.4-fold	

* In the case of items 10 through 14, only 61 animals were involved.

† The relative differences, expressed in percentage or in number of times the original amount, are based on the smaller of the two values.

‡ This was an exceptionally small animal; the next smallest in the entire group (48.25 gm) was in the same set (29).

denied that some differences could result from accidental differences in blood supply, placement in the uterus, etc., there seems to be scant basis for relating many of the large differences (there are 12 parameters for which the range is from 2-fold up to 140-fold) to such environmental influences. It seems much more likely that such differences are due to inescapable accidental differences in the makeup of the cytoplasms of the four primordial buds. These could be brought about, for example, by the segregation of mitochondria in the original egg.

It is recognized that epinephrine and norepinephrine levels in mammalian blood vary with the state of stress of the individuals, but since the newborn armadillos were handled in the same manner, the large differences in the contents of the spleens, brains, and adrenals could hardly be ascribed to the stress factor.

An interesting question in connection with this study is whether or not there is internal evidence in the data of the existence in the quadruplet sets of "two sets of two." Previous workers have speculated on this because of the way in which the primordial buds develop.

Quadruplet set 54 seems to exemplify this situation (Table 2). Armadillos 54A and 54B show resemblances to each other and contrast with 54C and 54D (which also resemble each other) in the case of 15 parameters. In the case of several parameters—adrenal weight, kidney weight, liver weight, small intestine length, epinephrine in adrenals, and aspartate, glycine, alanine, γ-aminobutyric acid (GABA), and taurine in the brain homogenates—the pairing off of these individuals appears striking. Evidence for "two sets of two" is much stronger in quadruplet set 54 than in any other set.

The findings summarized in Table 1 become vastly more important when we realize what kinds of characters may be associated with differences such as we have observed. As geneticists realize, the traits that can be linked to single

TABLE 2. *Data with respect to quadruplet set 54.*

	Body wt	Scute no.	Adrenal wt/100 gm	Brain wt/100 gm	Heart wt/100 gm	Kidney wt/100 gm	Liver wt/100 gm
A	60.30	526	0.345	5.864	0.577	0.943	3.269
B	57.19	529	0.350	5.225	0.446	0.970	3.263
C	55.40	529	0.546	5.233	0.581	1.588	3.974
D	52.61	531	0.633	5.413	0.641	1.440	3.804

Spleen wt/100 gm	Small inst. length/100 gm	Norepi-nephrine in spleen	Norepi-nephrine in brain	Norepi-nephrine in adrenals	Epinephrine in adrenals	Catecholamine, % as norepinephrine in adrenals
0.139	166	0.01	0.031	5.81	0.14	98
0.126	166	0.01	0.031	0.35	0.05	88
0.240	173	0.01	0.020	2.57	1.60	62
0.177	173	0.01	0.054	2.82	1.36	67

Aspartate in brain homogenate	Glutamate in brain homogenate	Glycine in brain homogenate	Alanine in brain homogenate	GABA in brain homogenate	Taurine in brain homogenate
6.49	20.57	9.93	12.29	3.92	7.80
5.12	15.86	8.07	10.76	2.24	5.16
1.69	16.11	3.63	4.92	7.78	16.07
1.17	12.24	3.30	3.33	5.16	10.01

genes are discontinuous. "The more deep-seated characters of a race, however, such as form, yield, intelligence, speed, fertility, strength, development of various parts, and so on, are in general characters which grade in quantity."[11]

The inheritance of characters which vary quantitatively "the most fundamental and important characters of the organism"[11] cannot be accounted for on the basis of single genes; hence if one assumes that genes are *the* carriers of inheritance, one must say that in these cases multiple genes are involved.

If, as we have postulated and as our experiments seem to support, the control of the extent of the proliferation of the various differentiated cells is controlled by extrachromosomal factors, then these factors, which may be thought of as "turning genes off and on," may be of the utmost importance in mammalian inheritance.

Form, intelligence, speed, fertility, strength, etc., are precisely the kind of characters which would be expected to be profoundly affected, if not controlled, by the factors which control the extent of proliferation of the differentiated cells. The extent of proliferation is something that might be expected to vary continuously.

Our experiments call attention to the probability that in mammalian genetics those influences which determine *how many* of each kind of cell and how much of each tissue will be produced during development are of paramount importance since they may govern "the most fundamental and important characters of the organism."

If our reasoning is valid, then it would conceivably be possible to be extremely well informed about and conversant with the genetics of single-cell organisms (where differentiation is not involved) and at the same time be completely ignorant about how the most fundamental characters are inherited in mammals, where the control of the extent of the proliferation of differentiated cells is perhaps a dominant phenomenon. Also, according to our evidence, it is erroneous to assume that monozygous human twins have identical inheritance.

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