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Studies on the Effects of Maintenance of Hamster Adrenals *in Vitro*[1]

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Abstract. Whole adrenals of the young adult hamster were maintained in culture. In most of the experiments, the organ was supported on lens paper, but, latterly, cellulose and gelatin sponge were used. The culture media employed were (1) Ringer's Solution (no nutrient), and (2) a nutrient mixture consisting of modified Krebs-Ringer's Solution, lactalbumen hydrolysate, yeast extract and horse serum. The maintenance period was up to seven days, and the culture medium was freshened regularly in certain cases. Organs were weighed at the beginning and end, and sometimes during maintenance. Histological appearance at termination was compared with that of a freshly extirpated adrenal.

In absence of nutrient, weight remained constant for the first day of culture, decreased over the following two to three days, and then levelled off at about 60 percent of its initial value. By contrast, organs cultured in the nutrient medium showed quite random variations in weight. There appeared to be no correlation between maintenance of weight and histological preservation; in point of fact, all organs which were well preserved after more than one to two days *in vitro* had lost weight considerably. Histological criteria are discussed.

Many reports have appeared of the successful culturing or maintenance *in vitro* of intact animal organs. Initial interest centered around embryonic organs and their subsequent growth and differentiation when cultured outside the organism. More recently, attention has been focused on the maintenance of organs from mature animals (e.g., Richter, 1958; Trowell, 1959) with a view to examining biochemical and physiological processes which occur under controlled environmental conditions. Trowell (1959) gives a concise survey of the accomplishments in organ culture since 1929.

This paper concerns the culturing *in vitro* of the adrenal gland of the adult hamster. The adrenal gland was chosen for this study because of its ease of extirpation and the availability of assay methods for testing its functional integrity (Schaberg and deGroot, 1958). To date, adrenal glands obtained from a variety of species, when cultured *in vitro,* have all shown early degenerative changes in the zona fasciculata (Trowell, 1959). The hamster was chosen because it is a known hibernator and its organs, of necessity, are exposed at times to large fluctuations in the internal environment. Since no culture medium duplicates the internal environment of an

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organism, any organ thus environmentally transplanted must possess a large capacity for adaptation if it is to survive intact. An organ from a hibernator was therefore considered a good choice for organ culture.

Due to the lack of information concerning culture of organs from this species, the choice of a proper nutrient would appear to be arbitrary. In order to ascertain whether any observed survival was attributable to the nutrient per se, or to the availability of a limited endogenous energy source, adrenals were cultured in both a balanced salt solution and the chosen nutrient.

The extent of survival of an organ, at the termination of the culture period, was assessed principally by its histological integrity. As an adjunct to this criterion, weight variations during the culture period were determined.

Materials and Methods

Male hamsters weighing approximately 90-120 grams were overdosed with chloroform or decapitated. Aseptic techniques were employed throughout the subsequent procedures. Each adrenal was removed, and the gland was placed in sterile Ringer's solution contained in a petri dish. Connective tissue was removed as completely as possible without disruption of the organ structure. Each organ was then placed in a separate vessel containing 5 ml. of the appropriate fluid and the supporting structure (as designated later under Experimental Variations). Each culture vessel, obtained from Bellco, consisted of a 1¾ in. diameter dish, equipped with a side arm and cover, having a total capacity of 10 ml. The side arm was stoppered with a rubber sleeve allowing for insertion of needles for any subsequent gassing or nutrient changes.

After ten minutes, when the organ had become thoroughly wetted, it was removed and weighed rapidly. Weights were obtained by placing the gland on a small piece of paper towel, which in turn was suspended at each end by a loop of wire and attached to the side arm of a torsion balance. When the weight was obtained, the organ was returned to the culture vessel. The weight of the paper towel, which had absorbed the nutrient surrounding the organ, was then noted and subtracted from the previous weighing.

The vessel was sealed with vacuum grease applied to the ground surfaces of the cover and dish and placed in an incubator maintained at 37° C. At the termination of the experiment each gland was reweighed, fixed overnight in Bouin's solution, embedded in paraffin, sectioned and stained with eosin-hematoxylin.

Experimental Variations

Maintenance in Ringer's Solution, Supported on Floating Lens

Tissue. Lens tissue (Arthur H. Thomas Co., No. 6325) was prepared according to the procedure described by Chen (1954) and floated on Ringer's solution. An organ was carefully placed at the center of each piece of floating lens paper. Seven hamster adrenals were cultured from one to seven days without changing or replenishing the fluid. Weights were also obtained, in some cases, at varying intervals. Five adrenals from adult rats were also cultured in this manner.

Maintenance in RLY + 10 Percent Horse Serum, Supported on Floating Lens Tissue. The portion of the nutrient designated RLY consisted of Rogers' modified Krebs-Ringer's buffered salt solution (Rogers, 1955) containing 0.4 percent glucose to which lactalbumin hydrolysate and yeast extract were added to final concentration of 0.5 percent and 0.1 percent, respectively. To this was added Horse Serum (Difco Labs.) to a final concentration of 10 percent. Both penicillin G and streptomycin were added to the medium in concentrations of 200 units and 200 g. per ml. of nutrient, respectively. The completed medium had a pH of 7.1. Twenty-one hamster adrenals were cultured in this nutrient up to six days. Some organs were weighed daily. The nutrient in some vessels was changed completely every two days, while other vessels were left undisturbed during the culture period.

Maintenance in RLY + 10 Percent Horse Serum, Supported on Cellulose Sponge. Rectangular blocks of cellulose sponge (DuPont Co., fine pore cosmetic sponge) measuring 1 $cm.^2$ x 0.4 cm. were employed to support the glands. The sponge was pretreated either according to the procedure outlined by Chen (1954) for lens tissue, or merely rinsed in glass-distilled water before being autoclaved. Two types of sponge block were prepared, one having a flat surface and the other grooved by two intersecting troughs cut 1-2 mm. deep and 1 mm. wide. Two adrenals were placed on flat-surfaced sponges and four placed in the troughs of the grooved sponges. The glands were cultured for three days, with either 2/3 of the nutrient changed every 24 hours, or the nutrient entirely changed every 36 hours.

Maintenance in RLY + 10 Percent Horse Serum, Supported on Immobilized Lens Tissue or "Gelfoam". Cellulose sponge blocks, 1 cm. x 2 cm. x 0.4 cm., through which a hole 0.5 cm. in diameter was bored, were employed to keep the lens tissue paper in a stationary position. The sponge blocks were saturated with the nutrient until the central hole was filled. Strips of lens paper 1 cm. x 3 cm., pretreated as before, were then placed over the sponge. An adrenal was placed on that portion of the lens tissue which covered the hole. "Gelfoam" blocks, (Gelfoam, No. 7859, Upjohn Co.) 1.5 $cm.^2$ x 1 cm., were prepared by saturation in the nutrient for a sufficient time for the full extent of swelling to occur. In this case, the amount

of nutrient was reduced from 5 ml. to 3-4 ml. so that the top of the sponge was not covered. Four hamster adrenals were maintained for three days, two on each type of support, with replacement of 2/3 of the nutrient every 24 hours.

Results

In the following description, "well preserved" indicates that the structure has retained the essential features visible in a control, freshly extirpated organ, viz., even staining of cytoplasm, cell borders well defined, nuclei readily visible, cortical zones well differentiated (Figure 5); "autolyzed" indicates that cytoplasm is granular, borders ill-defined, and many nuclei have undergone pycnosis; "somewhat autolyzed" represents an intermediate condition, while "severely autolyzed" refers to extensive blurring of cell borders, and almost total disappearance of nuclei.

Figures 3 and 4 show the gross histological appearance of fresh and severely autolyzed hamster adrenals, respectively.

The lens tissue used in these experiments was of a different make

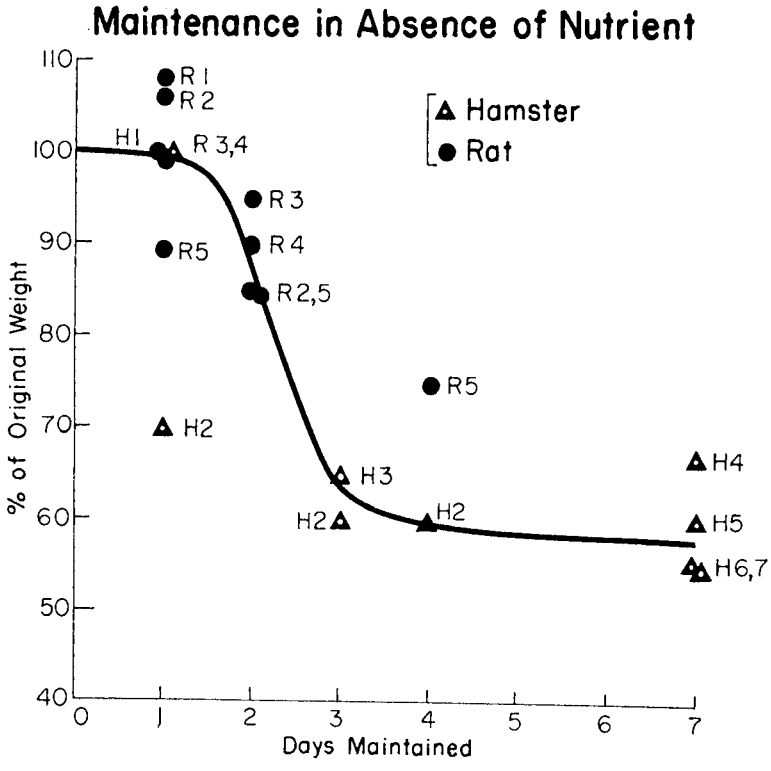

Figure 1. Relative weight variation of 5 rat and 7 hamster adrenals maintained in Ringer's solution.

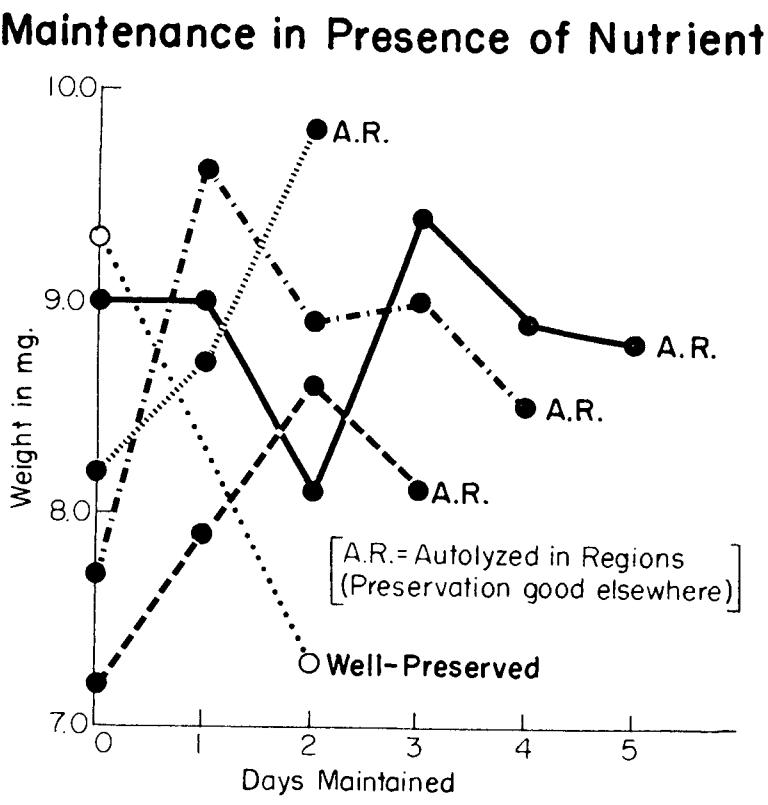

Figure 2. Weight changes and terminal histological condition of 5 selected hamster adrenals maintained in RLY + 10% horse serum. Straight lines are used merely to join consecutive weighings of an organ.

from that used by Chen (1954), and was found not to float consistently. Reference will be made to the sinking of organs where this is relevant to results obtained. Weighing of organs was not shown to be detrimental.

Maintenance in Ringer's Solution, Supported on Floating Lens Tissue. In no instance was an entire organ histologically well preserved for longer than one day. However, good preservation of the periphery was noted up to four days of culture. After from four to seven days culture, all portions of the organ showed signs of autolysis, although the cells were by no means entirely necrotic (compare Figures 6 and 7). Even the large rat adrenals were only somewhat autolyzed after four days *in vitro*.

The pattern of weight change was remarkably uniform with both hamster and rat adrenals (Figure 1). Weight was generally maintained for the first day, after which it decreased for 2-3 days, and leveled off at about 60 percent of its initial value.

Table 1
Maintenance of Hamster Adrenals *in Vitro*

No.	Weight relative to initial weight							Terminal Histological Condition	Means of Support
	1 day	2 days	3 days	4 days	5 days	6 days	7 days		
	Organs Cultured in Ringer's								
1	<–>							W	L.P.
2	–	–						P:W; C:A	L.P.
3	–	–						P:W; C:A	L.P.
4	–	–	↓					A	L.P.
5	–	–	–	–				P:W; C:A	L.P.
6	–	–	–	–				P:W; C:A	L.P.
7	↓	–	↓	↓				S	L.P.
8	–	–	–	–	–	–	↓	A	L.P.
9	–	–	–	–	–	–	↓	A	L.P.
10	–	–	–	–	–	–	↓	A	L.P.
11	–	–	–	–	–	–	↓	A	L.P.
	Organs Cultured in RLY + 10% HS								
12	↑							W	L.P.
13	↑	↑						R: W & A	L.P.
14	–	↓						W	L.P.
15	↑	↑	↑					R: W & A	L.P.
16	–	–	↓					R: W & A	L.P.
17	–	–	↓					R: W & S	Sponge
18	–	–	↓					R: W & A	Sponge
19	–	–	↓					R: W & S	Sponge
20	–	–	<–>					R: W & A	Sponge
21	↑	↑	↑	↑				R: W & A	L.P.
22	–	–	–	↓				R: W & A	L.P.
23	<–>	↓	<–>	<–>	<–>			R: W & A	L.P.
24	–	–	–	–	↓			R: W & A	L.P.
25	↓	↓	↑	↑	↑	↓		R: W & A	L.P.
26	–	–	–	–	–	↓		R: W & A	L.P.

Table 1 (Continued)
Maintenance of Hamster Adrenals *in Vitro*

No.	Weight relative to initial weight							Terminal Histological Conditions	Means of Support
	1 day	2 days	3 days	4 days	5 days	6 days	7 days		
				Organs Cultured in Ringer's					
27	–	–	–	–	–	<–>		A.Sev.	L.P.
28	–	–	–	–	–	↓		A.Sev.	L.P.
29	–	–	–	–	–	↓		A.Sev.	L.P.
30	–	–	–	–	–	↓		A.Sev.	L.P.
31	–	–	–	–	–	↑		A.Sev.	L.P.
32	–	–	–	–	–	↓		A.Sev.	L.P.
33	–	–	–	–	–	↓		A.Sev.	L.P.
34	–	–	–	–	–	↓		A.Sev.	L.P.
35	–	–	–	–	–	↓		A.Sev.	L.P.
36	–	–	–	–	–	↓		A.Sev.	L.P.

L.P. = Lens Paper
<–> = Initial Weight; ↑ Weight > Initial Weight; ↓Weight < Initial Weight
– = No weighing
W = Well preserved S = Somewhat Autolyzed A = Autolyzed
A. Sev. = Autolyzed Severely
P = Periphery of Organ C = Center of Organ R = Regions of Organ, (i.e., diversely scattered)

Maintenance in RLY + 10 Percent Horse Serum, Supported on Floating Lens Tissue. In no instance was an entire organ histologically well preserved for longer than two days. Organs which became entirely submerged during six days of culture were all autolyzed severely.

Weight variation appeared to be random (Figure 2). However, the one organ well preserved after two days *in vitro* had lost considerable weight.

Maintenance in RLY + Horse Serum, Supported on Cellulose Sponge. Organs supported on flat cellulose sponge were well preserved in their entirety after three days of culture. By contrast, organs maintained in troughs of the sponge showed areas of autolysis after the same culture period. The most severe autolysis occurred where sponges had not been pretreated according to the procedure employed for lens tissue.

All well-preserved organs had lost about 50 percent of their weight, whereas random variations in weight occurred with organs showing autolysis.

Maintenance in RLY + 10 Percent Horse Serum, Supported on Immobilized Lens Tissue or "Gelfoam". Organs cultured using these supporting structures were uniformly well preserved after three days. All organs had flattened considerably and had lost about 50 percent of their weight, but were histologically well preserved except for some shrinkage of cells (Figure 8).

Table 1 summarizes the data obtained for maintenance of hamster adrenals in all but the most recent experiments. Departures of less than 5 percent from initial weighing were considered insignificant, since the quantity of fluid transferred with the organ at each weighing was subject to a corresponding error.

Discussion

Cultures of the hamster adrenal for as long as three days without histological evidence of early degenerative changes in the inner cortex has not been reported by other workers (Schaberg, 1955; Richter, *et al.*, 1957; Trowell, 1959). Adrenals maintained by other techniques have been described to undergo changes in the zona fasciculata within two days of culture. Some of the obvious differences in techniques should therefore be noted. Important factors would seem to include the initial size of the organ, the nutrient employed, the gaseous phase, and any possible species difference.

In consideration of size, the radius of the organ would seem to be the most crucial factor because of its dependency *in vitro* on diffusion for exchange of gases and nutrients. Trowell (1959) states that

Figure 3. Hamster adrenal, excised, low power.

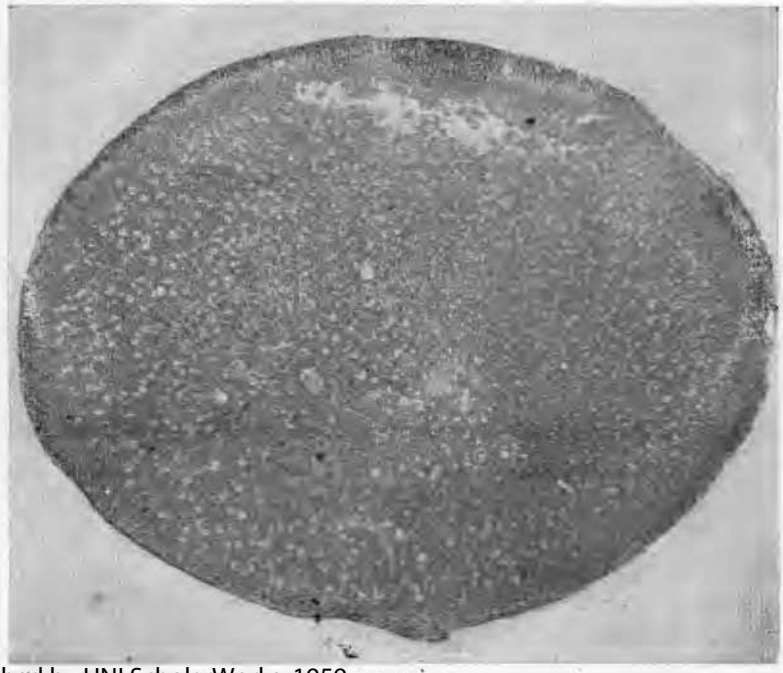

Figure 4. Hamster adrenal, severe autolysis, low power.

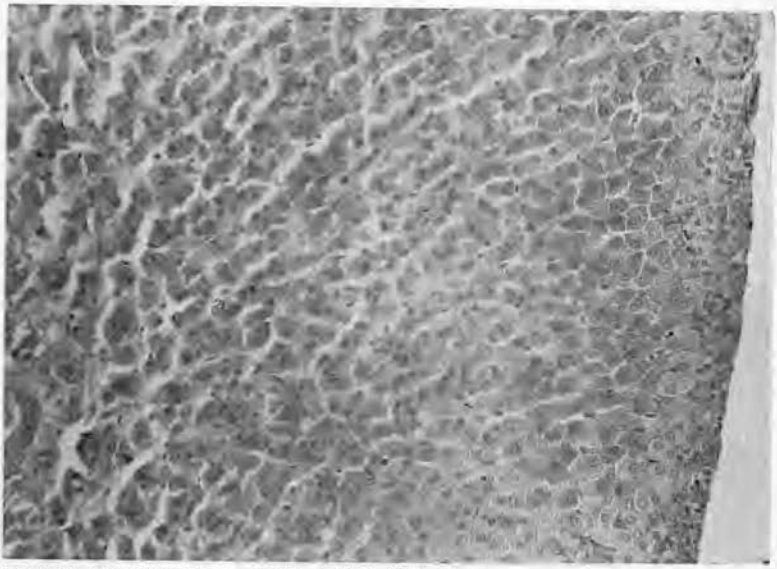

Figure 5. Hamster adrenal, freshly excised, high power.

Figure 6. Hamster adrenal, maintained 7 days in Ringer's solution. Autolyzed but not

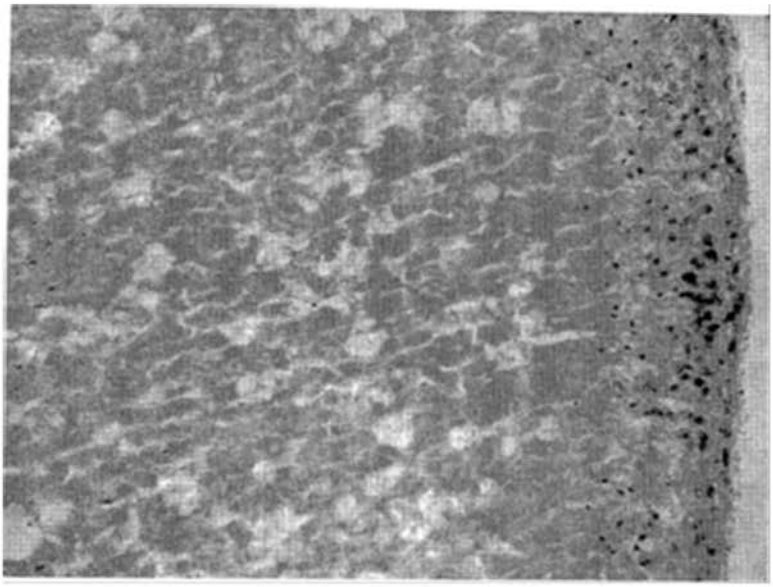

Figure 7. Hamster adrenal, maintained 6 days in RLY + 10 percent horse serum. Autolyzed severely.

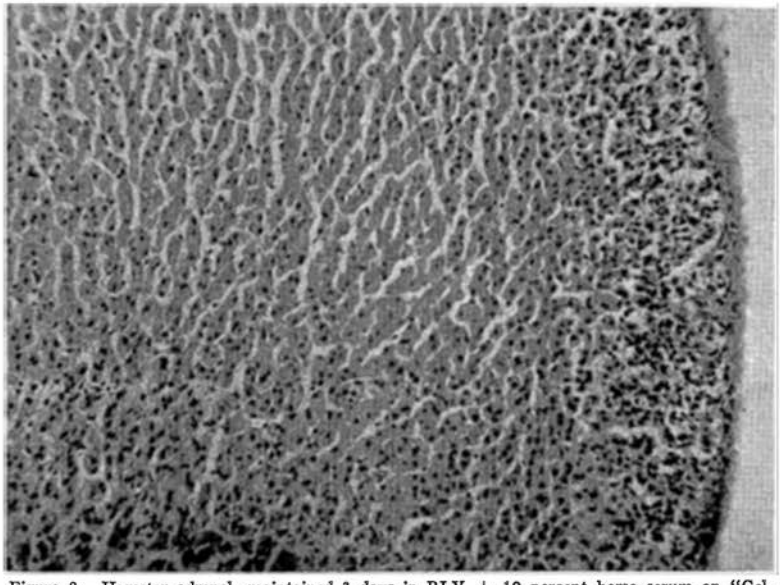

Figure 8. Hamster adrenal, maintained 3 days in RLY + 10 percent horse serum on "Gelfoam". Whole organ very well preserved. Some shrinkage of cells. (Poorly sectioned.)

spherical organs of larger diameter than 2 mm. cannot be maintained, even in oxygen, without the occurrence of central necrosis due to anoxia. However, both the adult hamster adrenals and Richter's bat adrenals exceeded this dimension. It is of note that all hamster adrenals which remained well preserved after three days had lost as much as 50 percent of their initial weight and had assumed a more flattened shape. Therefore, it would seemed plausible that the gland had reached dimensions within those specified for the maximum diameter. One might postulate that, at first, the organ would have been too large to obtain an adequate nutrient supply from the environment but could resort, to a limited degree, to an endogenous supply. If the maximum diameter were then attained before the endogenous supply was depleted, the organ could depend upon the external environment for its sustenance. The organs cultured in Ringer's solution, although quickly diminishing in size, could not resort to an exogenous nutrient supply.

The nutrient employed in this study was obviously utilized by the gland, since adrenals placed on floating lens tissue in nutrient displayed degenerative changes at a later time than those cultured in a similar fashion in Ringer's solution. However, one cannot ascertain if the nutrient was optimum. The use of a phosphate buffer system in the nutrient did result in remarkable constancy of pH as opposed to the marked fluctuations noted when organs are cultured in bicarbonate buffer systems. This stability of pH, over a three-day period, may have contributed considerably to the preservation of the organs.

It was also noted that organs were entirely preserved only when the bulk of their mass projected into the gaseous phase. It was for this reason that our attention turned to other means of support, when the brand of lens tissue that we were using did not remain floating over an extended period of time. Cellulose sponge (compare Cunningham and Estborn, 1958), when flat, "Gelfoam", and immobilized lens tissue, supported organs with a minimum of submersion. When even a fraction was submerged, as with the organ resting in a grove on cellulose sponge, regional autolysis was observed. In further consideration of the gaseous phase, Trowell (1959) found that high oxygen contents were necessary for the survival of adrenals from young rats. Our results, however, agree with the finding of Richter in that the hamster adrenal, as well as the bat adrenal, can be maintained in normal atmospheric air or even in the lowered oxygen tension which must prevail after a short culture period.

The differences in ease of maintenance of the adrenals cited may, of course, be entirely dependent upon species differences. The successful maintenance of an endocrine gland *in vitro* should prove to

be an important experimental tool for assaying the functional changes in the gland when exposed to imposed environmental changes. It is also hoped that use of the hamster adrenal gland *in vitro* may open another avenue of approach to a study of changes occurring during hibernation.

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