Table 1. Rectal glands of fresh-water and marine sharks. The fresh-water species were collected at Lake Nicaragua and Rio San Juan in June and July 1963, the marine species at Cape Haze, July to November 1963.

Animal No.	Sex	Body length (cm)	Rectal gland	
			Length (cm)	Weight (g)
	Fresh water: C. leucas			
1	F*	170	4.5	1.1
2	M*	158.4	6.5	0.6
3	F†	188.2	6.5	1.5
4	M†	174	6.7	0.6
5	F‡	155	6.0	0.8
6	M‡	147.5	6.4	0.7
7	F§	(127.2) ‖	6.0	0.35
8	F§	135	3.8	0.35
9	F§	157	4.0	0.4
10	F§	(165.6)	4.2	0.45
	Marine: C. leucas			
11	F	215.5		
12	F	231.0		
13	F	246.0	8.2	6.5
14	F	261.2	9.8	15.9
15	M	185.9	7.4	3.8
16	F	200.7	9.1	7.6
	Marine: C. limbatus			
17	M	142.5	3.2	1.5
18	F	161.3	5.4	3.0
	Marine: Galeocerdo cuvieri			
19	F	196.4	8.5	6.1
20	M	212.7	10.8	8.2
	Marine: Negaprion brevirostris			
21	M	265	7.3	12.0

* Collected at El Castillo. † Collected at San Carlos. ‡ Collected at San Juan del Norte. § Collected at Los Cocos near Granada. ‖ Not identified individually.

Galeocerdo cuvieri (two specimens), and lemon shark, *Negaprion brevirostris* (one specimen). Four rectal glands of marine sharks (Nos. 11, 12, 14, and 17 in Table 1) were fixed in Zenker-formol for histological examination. The rectal glands of fresh-water sharks were originally preserved in 70 percent ethyl alcohol, but they were fixed with Zenker-formol for routine histological procedures (*6, 7*).

Table 1 shows the measurements and other data on the rectal glands of bull sharks used in this study. For reference the data concerning three other species of marine sharks were included. A remarkable difference was observed in the size of rectal glands from marine bull sharks compared to those from Lake Nicaragua and Rio San Juan, even if the smaller size of the fresh-water sharks is taken into consideration. The weight of the rectal gland was greatly reduced, especially in four females from Los Cocos near Granada. In the histological preparations differences were also detected. The rectal glands of marine sharks are compound tubular glands, as shown in Fig. 1*A* which is a section from a female bull shark (No. 11 in Table 1). The cytoplasm of the excretory cells is granular, and it stained in the form of basal filament with eosin or phloxine. Cytological, histochemical, and electron microscope observations were made on the rectal glands of marine elasmobranchs by Bernard and Hartmann (*8*) and Doyle (*9*), and high activity in tubular cells was demonstrated morphologically.

The rectal glands from fresh-water sharks showed regressive changes. Figure 1 (*B* and *C*) shows the rectal glands of male and female sharks caught at El Castillo and San Carlos, respectively (Nos. 2 and 3 in Table 1). The glandular tubules are decreased in number, and the interstitial tissue between these tubules is increased in proportion. Some tubules are shrunken, and others are swollen. Laminated bodies resembling the casts and corpora amylacea found in renal tubules and prostatic ducts of man (*10*) are observed often within the lumina of these tubules (Fig. 1*C*). These bodies stain with PAS, iron-hematoxylin, phloxine, or chrome-hematoxylin, and are associated with tubular dilation and epithelial compression.

Thus, it seems that the rectal glands of Lake Nicaragua sharks become hypofunctional, or quiescent, and finally regressive changes associated with living in a fresh-water environment occur in their structure. These observations support the theory of Burger and Hess that the main function of the rectal gland is to excrete sodium chloride. It is not definitely known whether Lake Nicaragua sharks remain in the lake throughout life or whether they migrate back and forth between fresh water and the Caribbean Sea. The regressive changes were detected also in the rectal glands of sharks caught at the San Juan del Norte (Greytown), at the mouth of the Rio San Juan (Nos. 5 and 6 in Table 1). This suggests that they migrate (up and down) between Lake Nicaragua and the sea.

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7. The histological stains used are as follows: Ehrlich's acid hematoxylin and eosin; Gomori's chrome-hematoxylin and phloxine; Heidenhain's iron-hematoxylin and fast green; and periodic acid-Schiff (PAS) reaction.
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Mitotically Synchronized Mammalian Cells: A Simple Method for Obtaining Large Populations

Abstract. *Eliminating most of the ionic calcium from growth medium does not affect cell growth, but it allows simple and preferential detachment of HeLa cells in mitosis from nearly confluent monolayers.*

Biochemical studies of the mammalian cell in mitosis have been severely restricted by the lack of a simple system for achieving large populations in mitotic synchrony in spite of the critical role that biochemistry must play in eludicating the basic mechanisms of mitosis. Through environmental variations in temperature, or use of fluorodeoxyuridine (FUDR), some investigators have obtained what was termed parasynchronous division in mammalian cell cultures (*1*). In these systems a large proportion of the cells doubled their number within a short time interval relative to the total generation time. These techniques, however, are of little use for extensive investigation of the intracellular biochemical profile during the mitotic cycle because the actual increase in the number of mitotic figures present at any one time is usually less than 10 percent of the total population. Terasima and Tolmach (*2*) recently described the first significant improvement in the technique for obtaining mitotically synchronous mammalian cells. They have taken advantage of the relatively tenuous attachment of these

cells to the substrate, when grown in monolayers by selectively detaching them with gentle pipetting directed over the surface of the monolayer. In this way they were able to collect cells, 85 to 90 percent of which were in mitosis, while subjecting them to a relatively slight trauma. These authors have commented that shearing forces, induced by agitation, were ineffective in detaching the cells. Brief exposure to trypsin was also ineffective. In our experience with this technique we have essentially confirmed the results of Terasima and Tolmach but have found that pipetting the cells free from their attachments was not always productive and, in general, was impractical for collecting quantities adequate for extensive biochemical analysis.

Sinclair and Morton (*3*), using hamster cells, have found that this cell type required the comparatively drastic combination of cooling, trypsinization, and agitation to break its substrate attachment during mitosis. It is clear that if the binding forces between glass substrate and the mitotic cell could be decreased, a shearing force might then be effective in detaching the cells where it previously had failed. This would serve the threefold purpose of decreasing the time and manipulation necessary for cell collection, decreasing the cell trauma, and making an automatic collection system feasible.

Since the degree of cell-substrate binding is related to environmental Ca^{++} concentration, the critical modification which we have effected is the elimination of Ca^{++} from the growth medium except for that present in the serum supplement (0.0063 mg/ml, final concentration). Thus, HeLa S3 cells grown in Eagle's medium (*4*) with NaCl in osmotic equivalence substituted for $CaCl_2$ and supplemented with 7 percent fetal calf serum will spread on glass and multiply in a fashion not grossly different from cells growing in medium containing the normal complement of Ca^{++}. Thus, we use this medium routinely for indefinite periods of time without any noticeable morphological variation of the mitotic apparatus, chromosomes, or other cellular constituents. It is only for the determination of plating efficiency, where environmental requirements are more exacting, that the Ca^{++} concentration must be raised for optimal growth. However, when, and only when, the cells enter mitosis, their attachment becomes so tenuous that they detach when exposed to a minimal turbulence. Therefore, the harvesting procedure used to achieve synchronous populations is as follows: Monolayers are cultivated in Blake bottles (A. H. Thomas) in low Ca^{++} medium until they are nearly confluent. These large bottles contain about 4×10^7 cells. Two hours before use (less is inadvisable) the medium is replaced to eliminate any floating cells and debris. At collection time the bottle is grasped by the neck and shoulders, so to speak, held at eye level, and inverted with the monolayer facing the holder. The wrist and elbow are then flicked forward and down so that the medium, subjected to a centrifugal force, passes rapidly over the cells. One such movement is usually enough to detach most of the mitotic cells; care must be taken to avoid too rapid motion which may lead to detachment of some interphase cells. The bottle is quickly opened and the cell suspension is poured through fine nylon mesh (Tobler, Ernst and Traber) which has been spread over a 250 ml centrifuge cup to filter out any interphase clumps that may have been inadvertently shaken off. This filtration must be carried out by gravity only, since suction injures the cells and pulls through the undesired clumps. The cells are collected by gentle centrifugation. The average yield from a series of 20 confluent bottles is 30 to 40 mg of cells, as many as 95 percent of which are in mitosis. The bottles may be used every 30 minutes provided that the collection is carried out at 37°C. Several other strains of cell cultures, including human conjunctiva, L strain, and human epidermoid carcinoma, have been investigated and have been found to behave similarly. In addition, it may be noted that manipulation of the cells during the detachment process does not affect their plating efficiency; over 90 percent go on to divide and form clones.

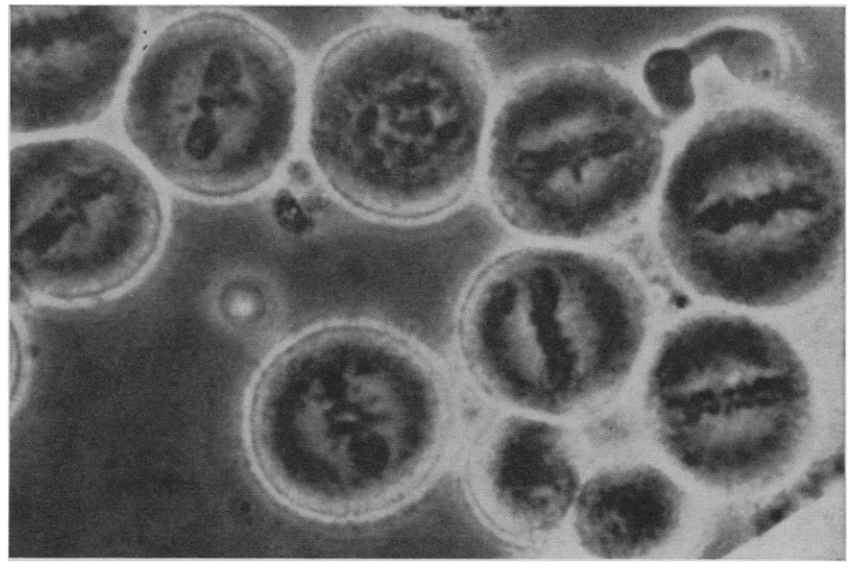

Fig. 1. HeLa cells synchronized in mitosis, obtained by preferential detachment from monolayers propagated in medium containing 0.0063 mg of Ca^{++} per milliliter (phase contrast, × 640).

Figure 1 shows that synchrony is virtually absolute, essentially all of the cells containing mitotic figures or just completing division. With this technique it thus becomes routine to collect up to ½ gram of synchronous cells per day and to carry out on the essentially untreated mammalian cell most, if not all, of the biochemical studies up till now only possible on synchronized populations of lower species (*5*).

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