specimens listed are probably all Pliocene in age.

All the specimens resemble Recent species of *Spheniscus*, and reference to that genus is sufficiently probable. It is improbable that all belong to a single species, because the available bones of the hind limb are distinctly larger, relative to the humeri, than in Recent species or in the few fossil species in which these bones are known in association. The femur, tibiotarsus, and phalanges are not readily identifiable to species in penguins and are especially poor for comparisons with fossils as no fossil species have them as holotypes. Those bones in the present collection could all belong to one species, which can best be designated as *Spheniscus* sp. indet. at present (see measurements of femora in Table 1).

Humeri are usually characteristic in penguins, and most of the taxonomy of fossil penguins is based on these bones, the tarsometatarsi (not available in the present case), or both. Specimen L6510 is smaller than any of the specimens of *S. demersus* with which it was compared: about 14 percent shorter than their mean and about 10 percent shorter than the smallest of them. It also has a slightly more sigmoid shaft and lacks the preaxial angulation, small but visible on all of five compared specimens of *S. demersus*. On specimen L12887A the length cannot be measured definitely, but it was probably about the length of specimen L6510 and was shorter than the compared specimens of *S. demersus*. The shaft is also somewhat sigmoid and without a preaxial angulation. However, the shaft is stouter than that of specimen L6510 and within the *S. demersus* range in this respect. The other humerus cannot be precisely measured except for distal width of shaft, but the preserved parts are virtually identical with specimen L12887A and also differ from specimen L6510 only in having a somewhat stouter shaft.

The fossil humeri differ from available *S. demersus* in three characters that are rather constant in penguins (*3*). The difference in length is significant ($P = < .05$, t-test) in spite of the small available sample of the Recent species (d.f. = 4). Although the distinction cannot be considered certain, it seems probable that the fossils should be distinguished specifically from the living penguins of the same region. There is nothing in the available data to preclude a directly ancestral relationship.

Table 2. Measurements, in millimeters, of humeri of fossil penguins from Langebaanweg and Recent *Spheniscus demersus*. A, Width of shaft, proximal, below internal tuberosity; B, width of shaft, distal, above distal expansion; C, maximum length; D, proximo-distal distance from most proximal part of head to below internal tuberosity.

Specimen	A	B	C	D	C/D	B/A
			Spheniscus predemersus			
L6510	9.5	10.8	~ 59¼	13.4	~ 4.4	1.14
L12887A	10.3	11.7				1.14
1/1968		12.0				
			Spheniscus demersus			
Range (4 specimens)	10.2–11.5	11.8–14.1	65.5–73.5	15.0–16.1	4.23–4.66	1.16–1.23
Mean (4 specimens)	10.80	12.80	69.10	15.60	4.43	1.18

Spheniscus predemersus, new species

Etymology: *Pre*, before, *demersus*, specific name of the living South African penguins, to indicate the earlier occurrence and probable relationship of the fossil species.

Holotype: South African Museum specimen L6510, as above.

Hypodigm: The type, L12887A, and humerus from site 1/1968, as above.

Known distribution. "E" quarry, Langebaanweg, late Pliocene of South Africa.

Diagnosis: Humerus shorter than in *S. demersus*, shaft more sigmoid and without preaxial angulation. The measurements are given in Table 2.

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7 January 1971

DNA Synthesis in Differentiating Skeletal Muscle Cells: Initiation by Ultraviolet Light

Abstract. *As skeletal muscle cells differentiate, they fail to initiate DNA synthesis. This rigid regulation, which persists even after cells are fully developed, does not extend to "repair" DNA synthesis, in that ultraviolet light initiates DNA synthesis in 99 percent of the muscle nuclei exposed. The rate of "repair" DNA synthesis in these nuclei, however, drops over 50 percent at the time of cell differentiation.*

The nuclei of multinucleated skeletal muscle cells have been shown never to enter the DNA synthetic phase of the cell cycle. These syncytial cells form in embryonic life by the fusion of many mononucleated cells which, until the time of fusion, are quite capable of DNA synthesis. Numerous attempts by autoradiography have failed to demonstrate incorporation of tritiated thymidine into nuclei of multinucleated skeletal muscle cells (*1*). The basis of the abrupt change from a proliferative to a nonproliferative cell at the time of cell fusion is not known.

Recent studies demonstrate both in vitro and in vivo that, as mononucleated cells fuse during muscle differentiation, they loose some 80 to 90 percent of their replicative DNA polymerase activity (*2*). Although, in eukaryotic cells, it is not known whether this particular DNA polymerase is involved, it is thought that the "repair" or "unscheduled" synthesis of DNA after damage by ultraviolet light does require a DNA polymerase (*3*). If the polymerizing enzyme involved in repair DNA synthesis is the replicative polymerase found in soluble fractions of eukaryotic cells, then one might expect to find either no repair synthesis or a deficiency in repair DNA synthesis in skeletal muscle cells as they go from the mononucleated to the multinucleated state of differentiation. The experiments reported here were performed to determine whether the controls of semiconservative DNA replication in

Table 1. Repair DNA synthesis in muscle cells. In experiment 1 mass cultures were exposed to ultraviolet light (150 erg/mm²) and then incubated in tritiated thymidine for 2 hours. Experiments 2 and 3 are clonal cultures in which the cells were preincubated 2 hours in tritiated thymidine. They were then treated with ultraviolet light (150 erg/mm²), followed by a 2-hour incubation in tritiated thymidine. Background grain counts have been subtracted in each case.

Experiment No.	No. of grains per 100 nuclei	
	Myotubes	Mononucleated cells
1	616	1308
2*	500	1200
3*	260	940

* Muscle cell clones.

differentiating muscle extend to repair synthesis (*4*).

Embryonic pectoral muscle tissue was removed from 11-day-old chick embryos and dissociated into single cells, which were plated as either mass or clonal cultures (*1*, *5*). At various times after plating, medium was removed from the dishes and they were exposed at room temperature to ultraviolet light (1.2 to 400 erg/mm²). Some dishes were preincubated in tritiated thymidine for 2 hours prior to treatment with ultraviolet light. All dishes received 5 to 10 μc of tritiated thymidine per milliliter of medium (specific activity, 13 to 23 c/mmole) after treatment with ultraviolet light. Autoradiographs were made from these dishes with the use of Kodak NTB-2 emulsion.

After 3 days of growth, mass cultures consist of two cell types—cells containing many nuclei, myotubes, and the mononucleated cells that have failed to fuse into multinucleated cells. Such cultures were exposed to various dosages of ultraviolet light and then to tritiated thymidine to detect repair DNA synthesis. The data in Figs. 1 and 2 show that one can initiate DNA replication in virtually every muscle nucleus with ultraviolet light and that the incorporation of tritiated thymidine is linear for 4 hours (*6*). The number of grains over activated nuclei of mononucleated cells is two or more times greater than the number over nuclei of multinucleated cells in the same population (Table 1). In order to demonstrate that this difference in the incorporation of tritiated thymidine is associated with the transition of a mononucleated cell to a multinucleated cell, muscle cells were cloned and subsequently exposed to ultraviolet light. Since all the cells in a muscle clone are daughters of a single cell, the difference in incorporation of tritiated thymidine can, therefore, be attributed to a difference in the state of differentiation of a cell type rather than to a mixture of different cell types in the population. Impaired repair synthesis is demonstrable in cloned cells as well and, therefore, is associated with the differentiative process.

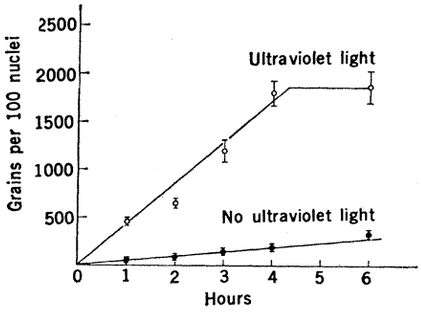

Fig. 1. Three-day mass cultures of muscle cells, growing in a mixture of minimum essential medium, horse serum, and embryo extract (88:10:2), were exposed to ultraviolet light (100 erg/mm²) and incubated in tritiated thymidine for the times indicated. Points represent the mean of the grain count ± standard error of the mean.

It is possible that these differences in incorporation of tritiated thymidine during repair are due to changes in pool size which occur as cells differentiate. To minimize this, clones of muscle cells were preincubated for 2 hours in tritiated thymidine in order to uniformly label the thymidine pools of mono- and multinucleated cells. The cells were then irradiated and again exposed to tritiated thymidine. The data in Table 1 demonstrate that, in preincubated cultures, cells still show a loss of repair synthesis associated with differentiation.

Ultraviolet damage to DNA is thought to result primarily in the formation of pyrimidine dimers, particularly those of thymine, within the single strands of double-stranded DNA. In bacterial systems it has been shown that these dimers are excised as a portion of an oligonucleotide (*7*), a process requiring at least two enzymatic steps; the missing region is replicated by a polymerase copying the remaining intact strand; and the process is completed by a ligase establishing continuity in the damaged strand. This is a nonconservative form of DNA replication. Although it is tempting to attribute the defect in repair synthesis to the loss of

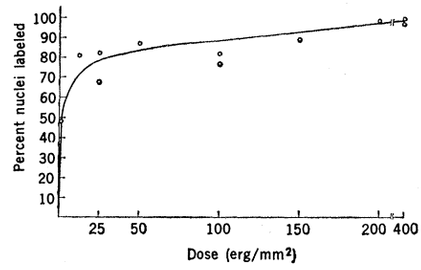

Fig. 2. Three-day mass cultures were exposed to ultraviolet light and then incubated in tritiated thymidine for 2 hours. Points are based on the percentage of myotube nuclei that exceed the mean grain count of the unirradiated control. Each point is based on counts of 100 or more nuclei.

DNA polymerase which occurs as muscle cells differentiate, any one or all of these steps may be defective in these cells. Cells from patients with the hereditary disease xeroderma pigmentosa have an impaired ability to repair ultraviolet damage, presumably because of a failure to excise thymine dimers (*8*), but these cells, unlike muscle cells, do replicate their DNA quite normally during the S phase of the cell cycle.

It is important to note that multinucleated muscle cells, at least during the early period after formation, must retain those enzymatic processes necessary for limited DNA synthesis and that this synthesis occurs in virtually every nucleus. The cells do this in spite of the fact that they never again would enter into the semiconservative replication of DNA. The rigid control exercised in this regard is found in few other cell types that retain nuclei. Incorporation of tritiated thymidine has been observed in myotube nuclei of cells infected with polyoma and Rous sarcoma viruses (*9*). In these studies it is unclear whether virus infection prevents the appearance of these rigid controls or initiates DNA synthesis subsequent to the appearance of normal controls. The uncertainty results from the apparent condition that only myoblasts, and not multinucleated differentiated cells, are directly subject to infection. In this ultraviolet light study, however, it is quite clear that nuclei in which the normal control processes have already appeared can be induced to reinitiate synthesis, albeit of a specialized nature.

In general, it has been thought that the availability of DNA polymerase has played no role in control of semicon-

servative DNA replication in the intact cell (*10*), but the work of Gurdon and colleagues on the activated amphibian egg, as well as the work done on the developing muscle system and the sea urchin, suggests that there may be instances in which DNA polymerase is involved (*2, 11*). How the observations reported here bear on this possibility is unclear because there are differences of several orders of magnitude between the amount of DNA synthesized in repair synthesis compared with S phase synthesis (*12*). Since the substrate requirements for repair synthesis must be minuscule compared to S phase synthesis, the remaining low levels of enzymes required for substrate formation and polymerization may be sufficient, albeit at a rate only 50 percent of that in precursor cells still in cycle. Or, the observations here may support the contention that a different enzyme or different enzymes are involved in the two types of synthesis.

These results demonstrate several things: (i) The controls of semiconservative DNA replication do not extend to repair synthesis; (ii) differentiated skeletal muscle cells can phosphorylate and incorporate thymidine into acid-insoluble material within each muscle nucleu; (iii) a polymerizing enzyme must be present in sufficient amount for this to occur; and (iv) this synthetic function (or functions) is partially lost during the process of differentiation. Whether this process requires the reversal of controls prohibiting replicative synthesis, or the maintenance or induction of a separate repair system, remains to be determined.

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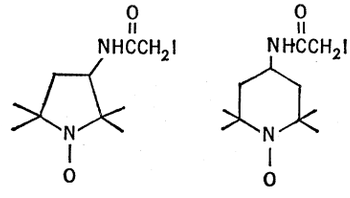
Conformational Equilibria in Spin-Labeled Hemoglobin

Abstract. *A component characteristic of deoxyhemoglobin appears in the paramagnetic resonance spectrum of spin-labeled oxyhemoglobin, and vice versa, under conditions of* p*H and ionic strength consistent with the interpretation that the spectrum is sensitive to the conformational equilibrium of the carboxy-terminal histidines. The oxygenation-induced change in the resonance spectrum is discussed in terms of shifts in this equilibrium.*

An oxygenation-induced change occurs in the paramagnetic resonance spectrum of hemoglobin that has been spin-labeled (*1*) at the 93β cysteine residues with either iodoacetamide-analog spin label **1** (*2*) or **2** (*3*).



1 **2**

Perutz *et al.* (*4*) and Moffat (*5*) have accounted for this change in terms of the oxygenation-induced shift in the conformational equilibrium of the COOH-terminus of the β-chains responsible for a major portion of the alkaline Bohr effect (*4*). The COOH-terminal (146β) histidine residues are free to take up several positions in oxyhemoglobin; in deoxyhemoglobin, hydrogen bonding of the imidazoles to the carboxyl groups of the 94β aspartate residues alters the protein conformation in the vicinity of the spin label at 93β and hence could perturb the resonance spectrum. We report results consistent with this interpretation and discuss previous observations on the basis of this conformational equilibrium.

Procedures for preparing spin labels **1** and **2** and for isolating and spin-labeling hemoglobin have been described (*2, 6, 7*). Conversion to deoxyhemoglobin was determined by the spectrophotometric method of Benesch *et al.* (*8*). An electron spin resonance (EPR) aqueous cell (Varian) was used for both optical and EPR measurements. Buffer *p*H was measured with a glass electrode (*9*).

Changes in the resonance spectrum of a spin label are usually interpreted in terms of changes in its tumbling rate (*1*). The effect of solvent viscosity on the resonance spectrum of compound **2** is shown in Fig. 1. As the solvent viscosity increases—as the tumbling rate of the molecule decreases—the "hyperfine separation" (*h* in Fig. 1) increases from 34 gauss when the molecule tumbles freely in solution to 68 gauss when it is immobilized (such a spectrum is commonly termed a powder spectrum).

The resonance spectrum of spin-labeled oxyhemoglobin (Fig. 2a), or that of methemoglobin, is not identical to that of unreacted spin label at any solvent viscosity. It is, in fact, a superposition of two spectral components due to labels with two different tumbling rates (*6*); there is a temperature- and buffer concentration–dependent equilibrium between these two states (*7*).

The low- and high-field features of each component (A and B) are indicated in Fig. 2a. Component A, with a hyperfine separation of 62 to 65 gauss (depending on buffer concentration), must be very nearly a powder spectrum (Fig. 1e) due to a conformational state (also denoted by A) in which the label is practically immobilized. Component B, with a hyperfine separation of about 40 gauss, is due to a state B, in which the motion of