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4. Gibrel is the name applied to the potassium salt of gibberellic acid by Merck & Co., Inc. The gibberellic acid used in these studies was prepared by the Chemical Division, Merck & Co., Inc., Rahway, N.J.
5. The tissues examined included skin, skeletal muscle, gonads and accessory sex organs, stomach, intestine, salivary glands, thymus, pancreas, adrenal, thyroid, parathyroid, lymph nodes, spleen, liver, kidney, urinary bladder, aorta, heart, lung, bone marrow, and usually the pituitary, brain, and spinal cord.

5 August 1957

Preparation of Cell-free Yeast Homogenate That Converts Acetate to Sterols

Cell-free yeast preparations have been applied recently to the study of sterol biogenesis (*1*). However, all these preparations require complicated apparatus for the mechanical disruption of yeast cells. A search for an easier method has been conducted in this laboratory over the last 2 years, and a method employing only the simplest equipment is described in this report (*2*).

Twenty grams of dry baker's yeast (Fleischmann) is suspended in 80 ml of 5 percent aqueous glycerol solution and stirred vigorously for 2 hours at room temperature with a Herschberg wire stirrer (Nichrome wire loops on a glass rod). The brei is then centrifuged at 1000 *g* for 30 minutes in the cold, and the supernatant is dialyzed against four changes of distilled water over a 24-hour period at 7°C to remove the glycerol. The homogenate (approximately 60 ml) contains particulate material but no whole cells or cell-wall debris. It is then diluted to 80 ml and divided into 20 Erlenmeyer flasks, each containing 1.0 mg of adenosine-5′-triphosphate (ATP), 1.3 mg of diphosphopyridine nucleotide (DPN), 1.6 mg of coenzyme A (CoA), 5 mg of methionine, 4 mg of $MgSO_4$, 8 mg of $NaNO_3$, 4 mg of K_2HPO_4, 2 mg of KCl, 0.04 mg of $FeCl_2$, and 20 mg of "tris" buffer (*p*H 7). Five microcuries of sodium acetate-1-C^{14} (0.41 mg) is added to each flask.

The incubation is carried out at room temperature in cotton-plugged flasks mounted on a rotary shaking table. After 48 hours, 4 ml of methanol and 0.8 g of potassium hydroxide pellets are added to each flask, and the mixture is hydrolyzed on a steam bath for 16 hours. The hydrolyzate is extracted with pentane, and the pentane phase is washed thoroughly with alkali. The sterols are isolated by precipitation with digitonin, followed by cleavage with pyridine and recrystallation. Radioactivity is measured in a gas-flow counter in which 1 μc is equivalent to 3×10^5 count/min.

Proper stirring of the yeast suspension in the glycerol solution is quite important. Gentle shaking on a rotary table produces weak homogenates. Suspensions digested with diammonium phosphate or treated in a Waring Blendor, in a Potter-Elvehjem or Virtis homogenizer or in a Hughes press also gave only weakly active extracts. Table 1 demonstrates the role of various cofactors in the system. The methionine requirement has been explored in experiments with methionine-methyl-C^{14}, which was found to yield ergosterol-28-C^{14} (*3*). Aeration during incubation is essential. Homogenates incubated under nitrogen gave only 17 percent of the yield in a comparable aerobic incubation. Potassium cyanide ($1 \times 10^{-3}M$), α,α-dipyridyl ($1 \times 10^{-3}M$), and digitonin ($1 \times 10^{-4}M$) strongly inhibit synthesis of sterols. In one experiment, varying quantities of sodium acetate were used to determine the capacity of the system to convert acetate into sterols, and it was found that 2 mg of acetate was the maximum that could be efficiently utilized by 4 ml of homogenate in 48 hours.

The duration of incubation determines the extent of incorporation of C^{14} into sterols. After 48 hours, one-third of the C^{14} in the nonsaponifiable fraction has been incorporated into sterols.

The homogenate remains active for a long time. In several experiments a small increase in incorporation of C^{14} into sterols was found even after a 96-hour incubation. Since the homogenate is apparently not a very good growth-supporting medium, proper care during its preparation is sufficient to prevent contamination in 90 percent of the cases. Passing it through a Seitz filter into sterile flasks prior to incubation eliminates the remaining contaminants. This has been verified by microscopic examination both before and after incubation. Contaminated flasks usually show a lower yield of sterols, probably because whole cells divert acetate to other uses.

Table 1. Cofactor requirements. Yeast homogenate was incubated for 48 hours at room temperature.

Cofactor	Total C^{14} in sterols (10^3 count/min)	Decrease in yield (%)
None	5.1	
Yeast hydrolyzate (30 mg)*	22.4	
Complete system	43.3	0
ATP omitted	24.5	43.5
DPN omitted	37.3	13.9
CoA omitted	39.7	8.2
Methionine omitted	22.4	48.4
$MgSO_4$ omitted	24.5	43.4

* Nutritional Biochemicals Corp., Cleveland, Ohio. It lacks any inherent enzymatic activity in this system.

Standing at 7°C for 24 hours prior to incubation does not materially affect the activity of the preparation, but standing in 5 percent glycerol solution decreases the efficiency of ergosterol synthesis.

GEORGE J. ALEXANDER
Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

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24 July 1957

Differentiation of Species by Paper Electrophoresis of Serum Proteins of Pseudemys Turtles

Serum proteins of a number of vertebrates, including the turtle, have been studied by paper electrophoresis, and differences between major groups have been noted (*1*). Several workers have used protein composition in taxonomic studies, employing precipitin or electrophoretic methods (*2, 3*). The present work (*4*) was undertaken to compare the serum proteins of closely related turtle species.

Striking differences were observed when 22 individuals representing three different species of the turtle genus *Pseudemys* were analyzed by paper electrophoresis. Included were three races of *P. scripta* (*scripta, elegans,* and *gagei*) from four widely separated localities (Florida, Kansas, Louisiana, and Mexico), *P. nelsoni* from Florida, and three races of the *P. floridana* complex (*floridana, suwanniensis,* and *mobilensis*) from two localities (Florida and Louisiana). While the representatives of the *P. floridana* complex are currently considered to be subspecies of a single species (*5*), the *floridana* and *suwanniensis* examples from Florida exhibit biological relationships characteristic of distinct species—namely, reproductive isolation in microgeographic sympatry (*6*). Further evidence presented in this report indicates a difference between the serum proteins of these two forms which substantiates a species level relationship (*7*).

Our paper electrophoresis techniques were the same as those described by Durrum (*8*); we used Spinco model R, series B apparatus; barbital buffer (ionic strength 0.05; *p*H 8.6); Heath Kit constant-voltage power supply (300 v d-c);

and a 5-hour run. Proteins were made visible with bromphenol blue and zinc sulfate, and lipoproteins with Oil Red O. Dyed protein strips were scanned and analyzed quantitatively with the Spinco Analytrol instrument. Twenty- to forty-microliter samples were applied on Whatman No. 3 MM paper strips. Blood samples were collected by severing a carotid artery and draining the blood directly into a 15-ml centrifuge tube. After the blood had clotted and had been centrifuged, a clear serum was obtained.

Figure 1 shows the paper electrophoretic patterns of the serum of four turtle forms, as well as the pattern of a sample of human serum for comparison. Table 1 gives the values for relative and total protein concentration, as determined with the Analytrol (*4*).

The results indicate that the fastest moving fraction of turtle serum (fraction V) has about the same mobility as human α_1-globulin. Fraction IV migrates to a position between α_2- and β-globulins. It thus appears that none of the turtle serum proteins is comparable to human serum albumin in its electrophoretic behavior. Another turtle serum protein (fraction II) has zero mobility, and a further fraction (I) exhibits apparently cathodic mobility, similar to human serum γ-globulin. Protein fraction III is common to all turtle sera examined and appears to be a lipoprotein upon staining with Oil Red O. This lipoprotein could be concentrated by dialyzing turtle serum against distilled water for 36 hours, when a precipitate formed; this precipitate, on electrophoretic analysis, appeared to be an immobile, possibly denatured, lipoprotein.

The most striking difference between the serum protein fractions of the four turtle forms examined is that *scripta* and *nelsoni* have a single band V, while *P. floridana* and *P. suwanniensis* exhibit double bands of the same electrophoretic mobility as band V. When less than 10 μl of serum was applied to the paper strip, band V was observed sometimes as a diffuse streak rather than as a distinct band or bands. The serum protein fractions of *scripta* and *nelsoni* are similar, except that the over-all protein concentration in the serum of *nelsoni* is higher than that in *scripta*.

Table 1. Relative and total concentration of turtle (*Pseudemys*) serum proteins as determined by Analytrol.

Species	Protein fraction (% of total)				Total (Analytrol units)
	I, II and III	IV	V	V′	
P. scripta	60.3	15.1	24.6		211
P. nelsoni	66.1	16.0	18.0		274
P. floridana floridana	76.2	9.9	7.3	6.5	353
P. f. suwanniensis	63.8	12.9	15.0	8.3	240

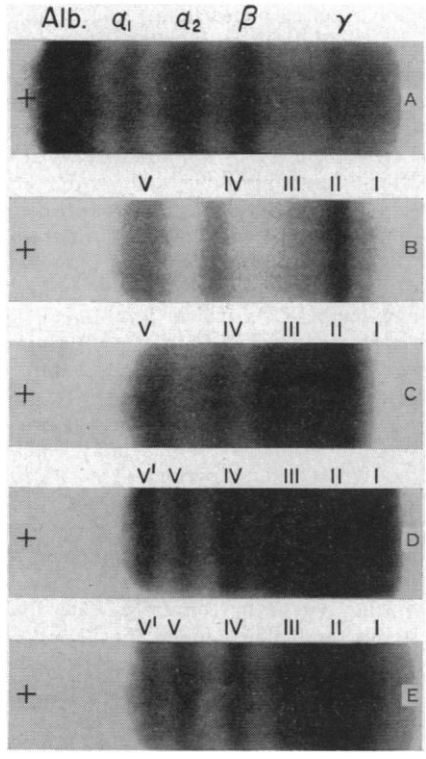

Figure 1. Paper electrophoresis patterns of serum proteins of turtles of the genus *Pseudemys* and of the serum protein of a human being (*A*) for comparison. Other patterns represent the species *scripta* (*B*) and *nelsoni* (*C*) and two subspecies, *floridana* (*D*) and *suwanniensis* (*E*), of the species *floridana*.

Only one example of *nelsoni* was available for these studies, and the observations regarding this form are tentative pending confirmation. In comparing the other two forms, it may be seen from Fig. 1 that protein IV in *floridana* has a greater relative concentration than V or V′, while fraction IV in *suwanniensis* appears (see Fig. 1) to be about equal in density to V and V′. Analytrol readings of *suwanniensis* show that IV is actually more concentrated than V′ but less than V. Fractions I, II, and III of *floridana* are usually more concentrated than the respective fractions of *suwanniensis*. The serum of one individual of *suwanniensis* showed two distinct bands III and III′, indicating the presence of two lipoprotein fractions which may be present but are not resolvable in the other animals studied.

Serum proteins of the nine examples of *scripta* exhibited no observable geographic variation. Slight variation in relative concentration and mobility of the lipoprotein fraction in different samples appears to be correlated with the age of the sample. In these studies it has been found that there are two basic protein patterns, one in which there is a single protein V (for example, in *nelsoni* and *scripta*), the other with two protein fractions V and V′ (for example, in *floridana* and *suwanniensis*). This would suggest that *scripta* and *nelsoni* may be more closely related to each other than to the *floridana* complex. Morphologic and zoogeographic evidence is not entirely consistent with this hypothesis.

The similarity of serum protein patterns of *floridana* and *suwanniensis* supports the conclusion of close relationship that is also indicated by other evidence (*5*). However, the observed difference in protein concentration between these two forms suggests that they are less closely related to one another than are the different subspecies of *scripta* which were examined. While the subspecies of *scripta* were indistinguishable, four examples of *suwanniensis* were clearly differentiable from three specimens of *floridana* from the same area on the basis of protein concentration. This observation, while consistent with the local species-level relationship of *floridana* and *suwanniensis*, would suggest that differences in protein concentration may evolve quite rapidly, as noted in these subspecies.

In Louisiana the subspecies *P. f. mobilensis* replaces *suwanniensis* ecogeographically and is sympatric with *floridana* just as *suwanniensis* is in Florida. However, reproductive isolation of *floridana* and *mobilensis* in Louisiana is not complete. A high incidence of hybridization has been reported in this area, and genetic introgression has been suggested (*6*). Of a series of four Louisiana specimens obtained, two were judged by morphologic, diagnostic characters to be most similar to *mobilensis* and two to *floridana*; however, other characters suggested hybrid ancestry. The serum protein patterns of the four animals were found to be undifferentiable from one another, exhibiting the pattern of *floridana*, including the dense band IV. This similarity supports the hypothesis of hybrid origin of these specimens.

McCabe and Deutsch (*3*), who used electrophoresis of proteins (bird egg white) as a taxonomic tool, found the method more sensitive for comparisons above rather than below the generic level. The results reported here suggest that the technique of paper electrophoresis may be effectively employed in intrageneric comparisons of some animal groups.

GUNTER ZWEIG*
C. F. Kettering Foundation, Yellow Springs, Ohio
JOHN W. CRENSHAW
Biology Department, Antioch College, Yellow Springs, Ohio

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* Present address: Pesticide Residue Research Project, University of California, Davis.

9 August 1957

Increase in Resting Membrane Potential of Skeletal Muscle Produced by Insulin

Although the mechanism of action of insulin is not known, evidence is accumulating to suggest that it alters the rate at which certain substances enter skeletal muscle. Stadie, Haugaard, and Vaughan (*1*) showed that insulin affixes itself firmly to muscle, perhaps to the muscle membrane. Levine, Goldstein, Huddlestun, and Klein (*2*) demonstrated increased movement of glucose and related hexoses into muscle in eviscerated animals, and Park and Johnson (*3*) confirmed the phenomenon in the rat. Fisher and Lindsay (*4*) made essentially the same observation in the isolated, perfused rat heart. These observations do not distinguish between an effect of insulin on muscle membrane and an effect on a hypothetical hexose transport system.

Recently use has been made of the fact that aldolase, an intracellular enzyme, diffuses from muscle incubated under a variety of conditions (*5*). Alterations in the rate of diffusion of aldolase are attributable to changes in membrane permeability; no transport mechanism need be invoked. Insulin increased the rate at which aldolase diffused from isolated rat muscle; this is presumptive evidence that insulin altered membrane permeability.

The sum of these observations suggests that insulin becomes associated intimately with the muscle membrane, perhaps deforming it to alter permeability. If this is true, the association between insulin and muscle membrane might lead to altered electrical properties of the membrane. From the well-known effect of insulin on serum potassium concentration, it was suspected that insulin might hyperpolarize the muscle membrane.

Insulin administered to the intact animal causes a decrease in concentration of potassium in serum owing at least in part to movement of potassium from extracellular fluid into muscle. The consequent increase in ratio of activities of intracellular and extracellular potassium should lead to hyperpolarization of the muscle membrane. However, there is no satisfactory explanation for the movement of potassium induced by insulin. It was conceivable that, rather than producing intracellular migration of potassium, which then caused a change in membrane potential, insulin might act by first hyperpolarizing the membrane; this, in turn, would lead to intracellular migration of potasium, impelled by the new potential difference.

For these reasons the effect of insulin on resting membrane potential of isolated rat skeletal muscle was examined (*6*). Insulin caused hyperpolarization of the membrane.

The test system was the peroneus longus muscle of the rat. The muscle was excised by freeing it gently and cutting its tendons without transection of muscle fibers. In young rats this muscle weighs about 25 mg and exhibits stable resting membrane potentials for several hours. The muscle was placed at rest length in an appropriate perfusion system containing balanced buffered saline-glucose-HCO_3 solution and K^+ at 4.7 milliequivalents per liter; it was then gassed with 95 percent O_2, 5 percent CO_2. Intracellular puncture was performed with KCl-filled microelectrodes of impedance of approximately 15 megohms. Signals were led through conventional circuitry and displayed on a cathode-ray oscilloscope at a scale of 25 mv/in. After the muscle had been probed widely and it had been determined that resting potentials were stable, insulin was added to the system; resting membrane potentials were measured from approximately the 20th to the 60th minute thereafter. In several experiments, as a control on changes with time, potentials were measured either in the absence of insulin or in its presence but not in both circumstances.

In 207 impalements in six muscles in the absence of insulin, resting membrane potential was 70 ± 0.7 mv (standard error of the mean; S.E.M.). In 320 impalements in six muscles in the presence of insulin, 0.1 to 0.3 unit/ml, resting membrane potential was 75.4 ± 0.5 mv (S.E.M.). Insulin produced an increase of 5.4 ± 0.89 mv (S.E.M.), a highly significant difference by t test ($t = 6$, $P \ll 0.0001$). Data from four paired experiments appear in Table 1. Insulin produced hyperpolarization, highly significant by t test, in all pairs.

The mean increase in membrane potential was 8 percent. Were this the result of movement of potassium from perfusion fluid to intracellular fluid, the ratio of activity of potassium inside muscle to that outside would be required to increase by about 50 percent, in accordance with the equation

$$\frac{E_R''}{E_R'} = \frac{\ln (K)_i''/(K)_o''}{\ln (K)_i'/(K)_o'} = 1.08$$

where E_R is the resting membrane potential, $(K)_i$ is the activity of potassium inside the cell, and $(K)_o$ is its activity outside the cell; single primes refer to control muscles and double primes to muscles after exposure to insulin. If the true mean increase were as small as two standard errors less than the observed mean increase in resting potential, the ratio of activities of potassium would have had to increase by about one-third.

Potassium concentration of perfusion fluid was, by design, held constant owing to the large volume of fluid, approximately 150 ml, compared to the small mass of muscle. Analysis of potassium in perfusion fluid by flame photometry demonstrated that its concentration was indeed constant.

Both peronei longi from a rat were removed. One muscle was placed in 150 ml of insulin-free perfusion fluid and gassed simultaneously with its mate in which membrane potentials were measured and which was exposed to insulin. At the end of the experiment the muscles were analyzed for potassium. In five pairs of muscles $(K)_i$ increased by 1, 14, 25, 27 and 44 percent. The greatest increase occurred in a muscle in which membrane potential increased by 14 per-

Table 1. Hyperpolarization of muscle membrane produced by insulin.

Insulin concentration (units/ml)	Fibers measured (No.)	Resting membrane potential*			
		Control (mv)	Insulin (mv)	Difference (mv)	P†
0.1	135	69.4 ± 1.6	75.4 ± 1.1	5.9 ± 1.9	< 0.01
0.3	124	70.6 ± 1.8	76.3 ± 1.0	5.7 ± 2.1	< 0.01
0.1	90	61.5 ± 1.0	68.5 ± 1.2	7.0 ± 1.6	< 0.001
0.1	58	71.7 ± 1.9	81.3 ± 1.5	9.6 ± 2.6	< 0.001

* Potentials are given as mean ± standard error of the mean. † P is probability that the difference measured occurred by chance, estimated by t test.