*DELINEATION OF CYSTINE AND CYSTEINE TRANSPORT SYSTEMS IN RAT KIDNEY CORTEX BY DEVELOPMENTAL PATTERNS**

By Stanton Segal and Inez Smith

DIVISION OF BIOCHEMICAL DEVELOPMENT AND MOLECULAR DISEASES, CHILDREN'S HOSPITAL OF PHILADELPHIA, AND DEPARTMENT OF PEDIATRICS, MEDICAL SCHOOL OF THE UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

Communicated by W. M. Krogman, April 21, 1969

Abstract.—The impaired ability of neonatal rat kidney cortex slices to take up L-cystine at a time when the ability to accumulate cysteine is similar to that of adult tissues indicates the separate nature of the transport processes for these amino acids. Dissimilarities in dependence on oxygen and temperature are also indicative of different transport systems. The intracellular form of the amino acid was largely cysteine when either cystine or cysteine was the transported substrate although significant amounts of both were incorporated into reduced glutathione. No difference in intracellular forms was found between neonatal and adult tissue.

Interest in cystine-cysteine interrelationships has been stimulated by two human inherited disorders: cystinosis,[1] where there is cystine storage in various tissues, and cystinuria,[2] where there is a renal loss of large quantities of L-cystine as well as L-lysine, L-arginine, and L-ornithine into the urine. Although the oxidized amino acid is stored in cystinosis and lost, in cystinuria, normally the intracellular form appears to be essentially all in the reduced form, cysteine.

The attention of this laboratory has primarily been focused on the complex situation that exists in the renal tubule cell of both normal and cystinuric humans as well as normal animals.[3–6] In this regard, an outstanding question has been whether there are separate transport mechanisms for cystine and cysteine in renal tubule cells. We have previously attempted to delineate differences in transport parameters for these two amino acids in adult rat kidney cortex slices, and although the results of these studies were indicative of separate systems, they were not conclusive.[6] Seeking a parameter associated with transport phenomena that would clearly distinguish between cystine and cysteine uptake mechanisms, we have examined the transport capabilities of kidney cortex from newborn rats for these compounds. This report demonstrates a distinction of the sulfur amino acid uptake mechanisms based on differences related to developmental patterns. The intracellular forms of the accumulated sulfur amino acids have also been determined in renal cortical cells from newborn and found to be similar to that of adult tissue.

Materials and Methods.—Chemicals: Cystine-S^{35} was purchased from Schwarz Bio-Research, Inc. By high-voltage electrophoresis[5] and thin-layer chromatography[7] various batches were found to contain 1–3% impurity migrating as cysteic acid. (Carboxy-C^{14}) inulin was obtained from New England Nuclear Corp. Dithiothreitol (DTT) and N-ethylmaleimide (NEM) were obtained from Calbiochem. Unlabeled amino acids were purchased from Nutritional Biochemical Co.

Incubation conditions: The cystine-S^{35} was made as a 2-mM solution in dilute NaOH containing about 45 μCi/ml. Solutions were made each week since analysis of these solu-

tions revealed oxidation to cysteic acid to occur with time. (Less than 1% oxidation occurred within the first week.) Ten μl of this solution plus unlabeled cystine was added per flask to give a final concentration of 0.07 mM. Dithiothreitol at a concentration of 2 mM was used routinely in the incubations to convert cystine-S^{35} to cysteine-S^{35} for the study of cysteine transport.[5, 8] When cystine was converted to cysteine by dithiothreitol, one half the amount of cystine was added to keep the final concentrations of cystine and cysteine equivalent. As found previously in adult tissue,[8] dithiothreitol did not alter the normal uptake of L-lysine, L-valine, or alpha-aminoisobutyric acid by kidney of newborn animals. The uptake of cysteine-S^{35} maintained in the reduced state by the addition of 25 mg of thiolated Sephadex[9] to each flask instead of DTT was the same as that observed with DTT as the reducing agent. Since the large particles of Sephadex are solely outside the tissue, the equivalent results with DTT indicated that intracellular DTT does not itself alter the cellular transport mechanisms for the sulfur amino acids.

As described previously, incubations were carried out in 30-ml plastic bottles in 2 ml of Krebs-Ringer bicarbonate buffer (KRB) pH 7.35 at 37° unless otherwise stated.[10] Conditions for anaerobiosis[10] sodium depletion[11] have also been reported previously.

Preparation of tissues: Adult kidneys were obtained from male Sprague-Dawley rats weighing 140–160 gm kept on water and Purina Chow ad libitum. Cortical slices were prepared with a Stadie Riggs microtome. Cortical slices could not be made with a microtome from kidneys obtained from rats up to 15 days of age. The best procedure for obtaining slices from newborn and other young kidney cortex was found to be the taking of cortical slices directly from the surface of the kidney freehand with a fine two-edged razor blade. These small slices from newborn kidneys weighing slightly over 1 mg were examined microscopically after fixation, paraffin imbedding, and hematoxylin and eosin staining of thin sections and found to be entirely cortical tissue of immature nature.

The small slices were made from the kidneys of an entire litter without sex distinction (usually three slices from each newborn kidney) and pooled. Three slices were taken at random from the pool and placed together in an incubation flask. In each experiment three such flasks were incubated for determination of each data point. Adult tissues were also incubated in triplicate, each flask containing a segment of a slice from each of three animals.

At the outset it was necessary to determine the proper adult cortical slice preparation with which to compare the surface segments made from newborn kidneys. Transport of several amino acids by small surface segments of cortex from adult rat kidneys was compared with that of microtome slices from the same kidney. In initial experiments the small surface segments from adult tissue which weighed less than 1 mg each were found to have much higher uptake of amino acids than slices and abnormally high extracellular fluid (ECF) compartments. This led us to examine the effects of the size of slices or segments on amino acid accumulation and ECF measurements. These results reported elsewhere established that segments of adult tissue weighing 1 mg or greater behaved as intact microtome slices with regard to establishment of concentration gradients and ECF compartment size.[12] Similar studies were carried out on newborn cortex, comparing small segments of tissue weighing 1 mg with larger segments weighing 6–7 mg prepared by "shelling" the cortex away from the medulla with a razor blade freehand. Identical accumulations of amino acids and the same ECF were found in both these newborn preparations. It thus appeared reasonable to assume that the 1-mg segments from young kidneys gave results representative of cortical transport for that age animal which could be related to that of adult microtome-prepared slices or segments of such slices weighing more than 1 mg each. Usually, the weight of the three segments from young animals in each incubation flask was from 3 to 6 mg, while segments of adult slices totaled 10–20 mg per flask.

Assay of tissue radioactivity: The technique for study of intracellular accumulation of isotopically labeled amino acids was essentially that described by Rosenberg, Blair, and Segal.[10] After the incubation, the tissue amino acid pool was extracted with warm water, the extracts and incubation media were assayed for S^{35} by liquid scintillation counting

techniques, and the ratio of counts per minute per milliliter of intracellular fluid (ICF) to counts per minute per milliliter of medium—$[S^{35}]_{in}/[S^{35}]_{out}$—was calculated according to the technique of Rosenberg, Blair, and Segal.[10] Similar ratios were obtained if the tissues were homogenized in 10% trichloroacetic acid and the resulting supernatants assayed for radioactivity.

The determination of the nature of the S^{35} compounds in the tissue was performed by homogenizing the tissues in 1 ml of 20 mM N-ethylmaleimide in phosphate buffer, 0.1 *M*, pH 7.4, as described previously to form cysteine and reduced glutathione adducts.[4] The deproteinized reaction mixture was then chromatographed on MN 300 cellulose thin-layer sheets (Brinkmann Instruments, Inc.) employing *n*-butanol/pyridine/acetic acid/H_2O (3/2/0.6/1.5) as solvent with appropriate cystine, cysteine-NEM, and glutathione-NEM standards according to the method of States and Segal.[7] The thin-layer sheets for each reaction mixture were cut into 1-cm segments and each segment was placed in 10 ml of liquid phosphor (Liquifluor, New England Nuclear Corp.) for assay in a liquid scintillation spectrometer of S^{35} in segments corresponding to cystine, cysteine-NEM, and glutathione-NEM.

Determination of extracellular space and total tissue water: The extracellular space of newborn and 5-day kidney cortex was found to be 23% of wet tissue weight by the method of Rosenberg, Downing, and Segal[13] with (carboxy-C^{14}) inulin. This value agrees with that observed by Webber and Cairns.[14] The ECF value for adult tissue has previously been determined to be 25%.[13] Total tissue water, determined as the difference between wet and dry weight, was found to be 78.5% of wet tissue weight for young tissue and 80% in adult tissue.

Efflux of S^{35} from tissues: Efflux of S^{35} from tissues was studied after preloading the slices by incubation with S^{35}-cystine and S^{35}-cysteine. The slices incubated with cystine were then transferred to new KRB buffer while those preloaded with cysteine were placed in KRB with 2 mM DTT to keep the sulfhydryl reduced. S^{35} was assayed in the new media at 3-min intervals as described in detail previously by Segal and Crawhall.[6]

Results.—Variation of cystine-S^{35} and cysteine-S^{35} uptake by kidney cortex with animal age: The uptake of cystine-S^{35} by renal cortex varied considerably with age of the animal. Figure 1*A* reveals that the ability to accumulate the S^{35} label in the intracellular fluid of newborn cortical cells is markedly impaired. In newborn tissue, the ratio $[S^{35}]_{in}/[S^{35}]_{out}$ increases very slowly with incubation time, reaching a value of 3 by 120 min. At 150 min (not shown) the value is also 3, indicating that the steady state has been reached by 120 min of incubation. These findings contrast markedly with the uptake by adult cortical cells where the ratio rises rapidly, at over twice the initial rate as the newborn and achieves a steady state of about 5. Five-day-old tissues function no better than newborn tissues, but by ten days of age the cortical cells show evidence of increasing ability to take up cystine. Fifteen-day-old tissue functions much like the adult. The addition of 5 mM glucose or succinate to the incubation media did not increase the rate of cystine uptake by newborn kidney cortex.

When cysteine-S^{35} was the transported substrate, newborn tissue showed no impairment of the uptake process and indeed, the intracellular S^{35} was greater than the adult tissue as the steady state was approached. Five-day-old tissue also showed a greater than normal $[S^{35}]_{in}/[S^{35}]_{out}$ in the steady state but less than the newborn, indicating that the adult tissue response was being approached with increasing age.

The nature of intracellular S^{35} after cystine and cysteine uptake: Employing the formation of NEM adducts to trap intracellular cysteine and high-voltage

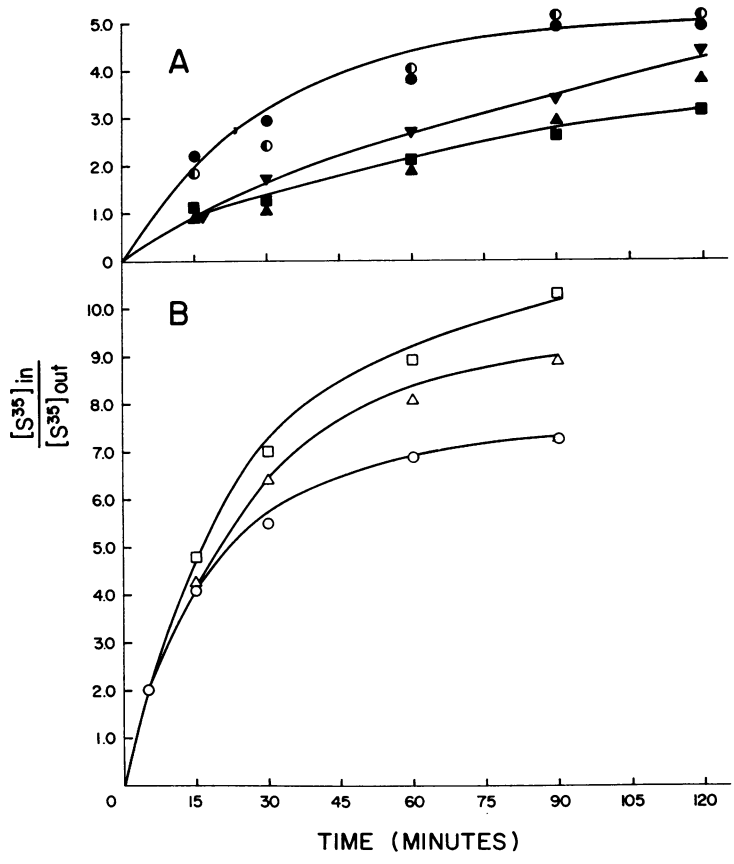

FIG. 1.—The uptake of cystine-S^{35} (*A*) and cysteine-S^{35} (*B*) by rat kidney cortex slices. Incubations described fully under *Materials and Methods* were carried out in Krebs-Ringer bicarbonate buffer pH 7.35 containing 0.07 μM/ml of the amino acid and 0.25 μCi/ml of label in a Dubnoff metabolic shaker. Flasks with cysteine contain 2 mM dithiothreitol. Solid and open circles represent tissues from adult animals; squares, newborn; triangles, 5-day-old; inverted triangles, 10-day-old; half-filled circles, 15-day-old. Each point is an average of from 6 to 20 determinations. $[S^{35}]_{in}/[S^{35}]_{out}$ is the ratio of counts per minute per milliliter intracellular fluid to counts per minute per milliliter of medium.

electrophoresis for the separation of cystine and the cysteine-NEM adduct in adult renal cortex, Crawhall and Segal[4] reported most of the S^{35} in the cells after cystine was transported to be in the form of cysteine. This has been examined again to determine if the intracellular forms after both cystine and cysteine transport differ in the young kidney (5-day-old) from the adult and thus could explain the variation of uptake with age. Table 1 shows that the intracellular forms of the S^{35} after transport are essentially alike in young and old tissue, except that there may be less glutathione-S^{35} in the adult with cystine as the substrate. Our newer thin-layer chromatographic technique[7] permits the assay of labeled glutathione which was not accomplished by high-voltage electrophoresis.[4] Quite substantial amounts of cystine and cysteine are incorporated into glutathione. The nature of the S^{35} in compounds other than those identified in the table is not

TABLE 1. *Intracellular forms of S^{35} after incubation of rat kidney cortex slices with labeled L-cystine and L-cysteine.**

Age of animal	Transported substrate	Concentration (mM)	No. of determinations	Intracellular Form of S^{35} as Per Cent of Intracellular S^{35}			
				Cystine	Reduced glutathione	Cysteine	Other
5 days	Cystine	0.07	3	0	25 ± 5	62 ± 5	13 ± 2
5 days	Cysteine	0.07	6	6 ± 0.5	24 ± 3	62 ± 2	8 ± 1
5 days	Cystine	0.5	3	11 ± 2	30 ± 3	51 ± 1	8 ± 2
5 days	Cysteine	0.5	3	8 ± 0.5	19 ± 1	68 ± 5	5 ± 2
Adult	Cystine	0.07	5	0	12 ± 2	68 ± 3	20 ± 3
Adult	Cysteine	0.07	5	14 ± 2	20 ± 4	64 ± 5	8 ± 3

* Incubation conditions and technique for determination of intracellular forms are described under *Materials and Methods*. A correction has been applied to the values to account for S^{35} substrate which is in the extracellular fluid.[5] Values are means ± SE.

known. The radioactivity designated in the "other" column does not represent discrete peak areas on the chromatograms, but is a summation of all the counts lying between the cystine, cysteine-NEM, and glutathione-NEM areas.

The intracellular nature of the S^{35} was also probed after incubation of slices with 0.25 and 0.5 mM cystine-S^{35} in order to determine if the slow cystine uptake in young tissues may be due to diminished intracellular reduction or conversion. These data are shown in Table 1. Only at 0.5 mM cystine was there a slight impairment of reduction as evidenced by 10 per cent intracellular S^{35} as cystine, thus indicating that at 0.07 mM, the concentration routinely employed, reduction is not a limiting process.

Efflux of S^{35}: The intracellular radioactivity in the system studied here is dependent not only on influx rate, but also on efflux rates. Thus, an impaired ability to accumulate S^{35} compounds by newborn renal cortex incubated with cystine could be due to impaired influx, accelerated efflux, or both. The slow uptake early in the incubation suggested that the influx mechanism was deficient. This was borne out by performance of S^{35} efflux studies as described previously.[6] Both five-day-old and adult cortex were incubated for 60 minutes with cystine-S^{35} at substrate levels such that the intracellular S^{35} was the same. The tissues were then transferred to buffer and the S^{35} in the medium assayed at 3-minute intervals for 18 minutes. The efflux rate constant for the S^{35} did not differ for the two tissues.

On the other hand, while cystine influx seems impaired in the young tissue, cysteine-S^{35} accumulation is higher in young tissue. From the curves of Figure 1*B*, it appears that cysteine-S^{35} influx may be accelerated and account for the finding of elevation of the steady-state intracellular S^{35}. The efflux of S^{35} into KRB containing DTT was found to be the same in newborn and adult tissue, thereby indicating that an increase in influx rate underlies the increased cysteine uptake in the newborn.

Effects of anaerobiosis and sodium deprivation on uptake of cystine and cysteine by cortex from five-day-old animals: Figure 2 shows the effects of anaerobiosis and sodium deprivation on sulfur amino acid uptake. In contrast to the profound inhibition of uptake of these amino acids due to lack of oxygen in the adult kidney cortex,[6] there is no impairment of the already slow uptake of cystine in

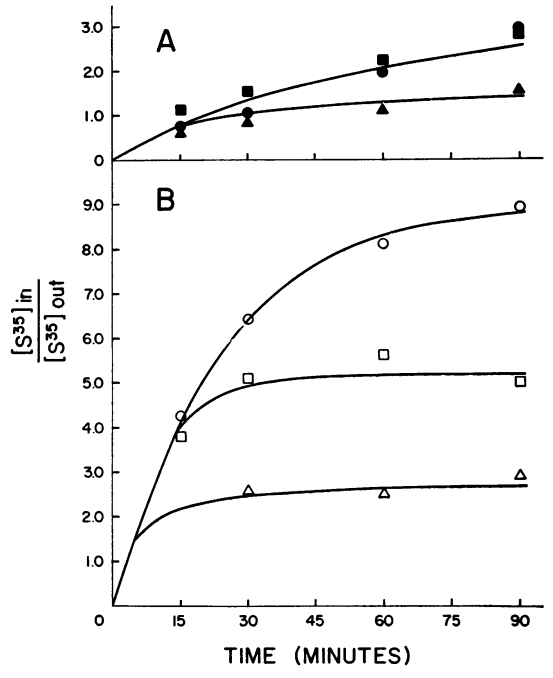

FIG. 2.—The effect of anaerobiosis and sodium deprivation on the uptake of cystine-S^{35} (*A*) and cysteine-S^{35} (*B*) by rat kidney cortex slices from 5-day-old animals. Circles represent control flasks; squares signify anaerobic conditions with an atmosphere of 95% N_2–5% CO_2; triangles indicate that sodium-free buffer in Na^+ was replaced by Tris. Each point is an average of three to six determinations.

young tissue and only moderate impairment of cysteine uptake. As in the adult,[6] sodium deprivation markedly inhibits uptake of both amino acids.

Table 2 shows the nature of the intracellular S^{35} when cysteine is transported under anaerobic incubation conditions. With oxygen lack, there is almost complete cessation of the radioactivity being incorporated into glutathione.

Effect of temperature: The uptake of cystine-S^{35} and cysteine-S^{35} was studied in five-day-old tissues at 20°. The results are shown in Figure 3. The slow uptake of cystine was markedly impaired at the reduced temperature. Cysteine uptake was essentially unaffected by the low temperature. These results are quantitatively similar to those seen in adult tissue.[6] Analysis of intracellular S^{35} after cysteine uptake at 20° revealed a diminished incorporation of the label into glutathione (Table 2).

Discussion.—The previous comparison of the parameters of cystine and cysteine uptake by adult rat kidney cortex slices indicated that separate mech-

TABLE 2. *Intracellular forms of S^{35} after incubation of kidney cortex slices of five-day-old animals with L-cysteine-S^{35} under anaerobiosis and reduced temperature.**

Temperature (°C)	Condition	Intracellular Form of S^{35} as Per Cent of Total Intracellular S^{35}			
		Cystine	Reduced glutathione	Cysteine	Other
37°	Aerobic	5 ± 0.3	30 ± 2	59 ± 1	6 ± 2
37°	Anaerobic	5 ± 0.3	6 ± 0.3	84 ± 0.5	5 ± 0.6
20°	Aerobic	6 ± 0.1	15 ± 1	75 ± 1	4 ± 0.5

* Incubation conditions and technique for estimation of intracellular forms is described under *Materials and Methods*. Anaerobiosis was produced by an atmosphere of 95% N_2–5% CO_2. A correction was made for substrate in the extracellular fluid of the tissue. Triplicate determinations mean ± SE.

anisms existed for mediating the movement of these amino acids into cells. The present data, showing that cystine entry in the newborn cortex is impaired while that of cysteine is not, clearly demonstrate the individual nature of the uptake process for these substances. The situation in the newborn cortex is analogous to that observed in the intestinal mucosa of human cystinuric subjects where there is a defect in cystine, but not in cysteine, transport.[15]

The transport processes for each of the sulfur amino acids in the newborn have characteristics similar to those observed in the adult. Sodium dependence and the responses to decrease in temperature are the same. The uptake by young tissues is not as dependent on aerobic conditions, which probably reflects the general property of resistance to anoxia in young tissues.

The slow uptake of cystine by newborn cortex does not appear to be related to the intracellular reductive fates of the amino acid. The patterns of intracellular metabolites in newborn and adult cortex are essentially the same. Moreover, we have demonstrated that at the substrate concentration employed in our experiments, 0.07 mM, there is no limitation of the reductive processes. How the reduction of cystine is related to the transport of the amino acid is not entirely understood. Preliminary data in this laboratory indicate that in the intestinal mucosa, which behaves like the kidney cortex in having reduced cystine in intracellular fluid, the reductive process is not located in the brush border but is a process taking place in the soluble cell contents.[16]

The present data therefore indicate that the cystine transport mechanism itself undergoes a maturation with age while that for cysteine is mature at birth. Ultramicroscopic studies have shown that the microvillae of the kidney tubule cell of the rat are not totally formed at birth but subsequently take on an adult appearance.[17] It seems that the uptake process for cystine may depend on a normal adult microvillous structure, but that for cysteine uptake does not.

Hyperaminoaciduria has been observed in the immature rat[18] and human.[19, 20] In the immature human, cystine appears in urine in larger quantities than in the

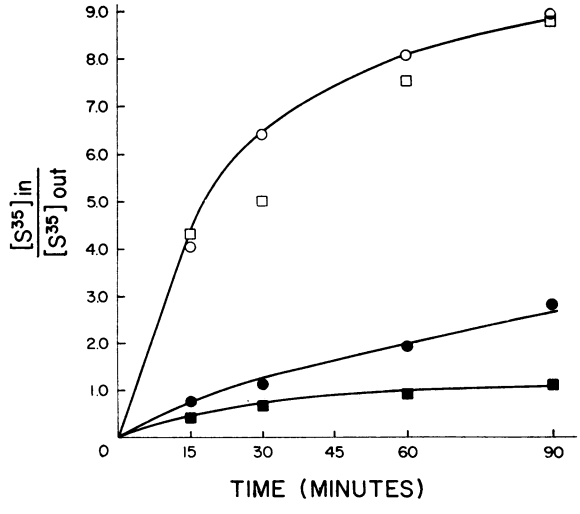

FIG. 3.—Effect of temperature on uptake of cystine-S^{35} (*solid symbols*) and cysteine-S^{35} (*open symbols*) by kidney cortex slices from 5-day-old animals. Circles signify control incubations at 37°C; squares represent incubations at 20°C.

adult.[20] Clearance data and levels in urine, however, may be difficult to interpret and relate to *in vitro* observations. Urinary data are a summation of many functions within the kidney tubule. It may well be that in the immature animal, cystine reabsorption is dependent on the cysteine transport system, while in the adult this is not the case. Our findings suggest the need of differential physiological studies of cystine and cysteine handling by the kidney of immature animals.

* Supported by grants from the John A. Hartford Foundation and the USPHS (AM10894).

[1] Seegmiller, J. E., T. Friedman, H. E. Harrison, V. Wong, and J. A. Schneider, *Ann. Internal Med.*, **68**, 883 (1968).

[2] Arrow, V. K., and R. G. Westall, *J. Physiol. London*, **142**, 41 (1958).

[3] Fox, M., S. Thier, L. Rosenberg, W. Kiser, and S. Segal, *New Engl. J. Med.*, **270**, 556 (1964).

[4] Crawhall, J. C., and S. Segal, *Biochem. J.*, **105**, 894 (1967).

[5] Segal, S., and J. C. Crawhall, *Biochem. Med.*, **1**, 141 (1967).

[6] Segal, S., and J. C. Crawhall, these PROCEEDINGS, **59**, 231 (1968).

[7] States, B., and S. Segal, *Anal. Biochem. Exptl. Med. Calcutta*, in press.

[8] Crawhall, J. C., and S. Segal, *Biochim. Biophys. Acta*, **121**, 215 (1966).

[9] Eldjarn, L., and E. Jellum, *Acta Chem. Scand.*, **17**, 2610 (1963).

[10] Rosenberg, L. E., A. Blair, and S. Segal, *Biochim. Biophys. Acta*, **54**, 479 (1961).

[11] Fox, M., S. Thier, L. E. Rosenberg, and S. Segal, *Biochim. Biophys. Acta*, **79**, 167 (1964).

[12] Smith, I., and S. Segal, *Biochim. Biophys. Acta*, **163**, 281 (1968).

[13] Rosenberg, L. E., S. Downing, and S. Segal, *Am. J. Physiol.*, **202**, 800 (1962).

[14] Webber, W. A., and J. A. Cairns, *Can. J. Physiol. Pharmacol.*, **46**, 165 (1968).

[15] Rosenberg, L. E., J. C. Crawhall, and S. Segal, *J. Clin. Invest.*, **46**, 30 (1967).

[16] States, B., and S. Segal, *Biochem. J.*, **113**, 443 (1969).

[17] Suzuki, Y., *J. Electronmicroscopy Tokyo*, **6**, 52 (958).

[18] Weber, W. A., *Can. J. Physiol. Pharmacol.*, **45**, 867 (1967).

[19] Soupart, P. in *Amino Acid Pools*, ed. J. T. Holden (Amsterdam: Elsevier Publishing Co., 1962), p. 220.

[20] Stambaugh, R., D. T. Davidson, Jr., and J. R. Elkinton, *Clin. Chem.*, **9**, 210 (1963).