Enzymatic Desulfination of Cysteine Sulfinic Acid*

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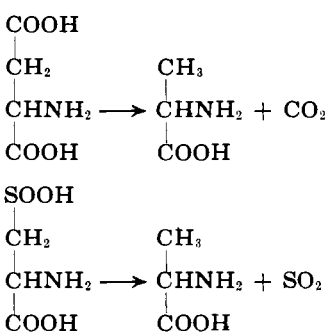
Highly purified aspartate β-decarboxylase from *Alcaligenes faecalis* catalyzes the conversion of cysteine sulfinate to stoichiometric amounts of sulfite and alanine. The ratio of the aspartate β-decarboxylase and cysteine sulfinate desulfinase activities was constant throughout purification from the initial cell sonicate to the final preparation. Desulfination is activated by α-keto acids and by pyridoxal 5′-phosphate in a manner similar to that observed for aspartate β-decarboxylation. Preincubation of the enzyme with L-aspartate led to loss of desulfinase activity and preincubation with L-cysteine sulfinate destroyed aspartate decarboxylase activity; neither activity was lost when α-ketoglutarate was added to the preincubation mixtures, and both activities were restored by addition of pyridoxal 5′-phosphate. The findings indicate that activation of desulfination by α-ketoglutarate is due to transamination of enzyme–pyridoxamine phosphate to yield enzyme–pyridoxal phosphate and glutamate as previously demonstrated for aspartate decarboxylation. The ratio of the rates of desulfination to transamination under the conditions employed is about 600. Desulfination is competitively inhibited by aspartate. Although the enzyme exhibits greater affinity for aspartate than for cysteine sulfinate, desulfination occurs more rapidly than decarboxylation when saturating concentrations of substrate are used.

In the course of studies on the β-decarboxylation of aspartate (Novogrodsky *et al.*, 1963; Novogrodsky and Meister, 1964), it occurred to us that aspartate β-decarboxylase might catalyze an analogous direct desulfination of cysteine sulfinate. We have investigated this possibility and found that the highly purified aspartate β-decarboxylase of *Alcaligenes faecalis* catalyzes the conversion of cysteine sulfinate to alanine and sulfite.



The enzymatic desulfination reaction, like the β-decarboxylation of aspartate, is markedly activated by both α-keto acids and pyridoxal 5′-phosphate. The data indicate that activation of desulfination by α-keto acids is due to transamination of enzyme-bound pyridoxamine phosphate, and that activation by pyridoxal 5′-phosphate is due to reconstitution of the apoenzyme as shown previously for the decarboxylation of aspartate. The present studies indicate that the same enzyme catalyzes both reactions.

EXPERIMENTAL

Materials.—L-Cysteine sulfinic acid was purchased from Mann Research Laboratories, Inc. The other compounds were obtained as previously described (Novogrodsky and Meister, 1964).

Purification of the Enzyme.—*Alcaligenes faecalis* (strain N) was cultivated and aspartate β-decarboxylase was isolated from this organism as previously described (Novogrodsky and Meister, 1964) with the following modifications. In a preparation of the enzyme from 30 g of dried cells, the precipitate obtained by addition of ammonium sulfate to 50% of saturation was dissolved in 10 ml of 0.1 M potassium phosphate buffer (*p*H 6.7) containing 10^{-4} M pyridoxal 5′-phosphate, and this solution was passed through a Sephadex G-200 column (2.5 × 190 cm) prepared in 0.005 M potassium phosphate buffer (*p*H 6.7). The enzyme was eluted with the same buffer and the fractions containing the enzyme were combined and added to the top of a DEAE-cellulose column. The column (2.5 × 40 cm) was prepared in 0.005 M potassium phosphate buffer (*p*H 6.7) and was developed with a linear gradient between this buffer and a 0.5 M potassium phosphate buffer of the same *p*H. Fractions containing 9.2 ml each of effluent were collected; those containing the enzyme were combined and the protein was precipitated by addition of sufficient solid ammonium sulfate to yield 100% of saturation. After centrifugation the enzyme was dissolved in 0.1 M potassium phosphate buffer (*p*H 6.7) containing 4×10^{-4} M pyridoxal 5′-phosphate and incubated at 37° for 30 minutes. It was then dialyzed against 0.1 M potassium phosphate buffer (*p*H 6.7) for 18 hours at 5°.

Methods.—Aspartate β-decarboxylase was determined essentially as described previously (Novogrodsky and Meister, 1964) by measuring the evolution of carbon dioxide in a Warburg manometric apparatus. The standard assay system consisted of sodium L-aspartate (15 μmoles), sodium α-ketoglutarate (0.5 μmole), pyridoxal 5′-phosphate (0.5 μmole), sodium acetate buffer (260 μmoles), and enzyme in a final volume of 1.5 ml. The final *p*H was 5.0. The mixtures were incubated for 15–60 minutes at 37°. Desulfinase activity was determined in the same system except that sodium L-cysteine sulfinate was substituted for sodium L-aspartate. Sulfite was determined by the fuchsin method of Grant (1947); the colors were compared at 550 mμ. The formation of alanine was determined by paper chromatography on Whatman No. 1 paper in a solvent consisting of 1-butanol–acetic acid–water (4:1:1). The reaction mixtures were deproteinized by addition of three volumes of cold ethanol followed by centrifugation. After chromatography the spots corresponding to alanine and a series of simi-

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TABLE I
PURIFICATION OF THE ENZYME

Fraction	Volume (ml)	Total Protein (mg)	Specific Enzymatic Activities[a] Desulfinase (A)	Decarboxylase (B)	Ratio B/A
Cell sonicate	290	2840	18	35.4	2.0
$(NH_4)_2SO_4$ fraction (0–30%)	30	80	68	154	2.3
$(NH_4)_2SO_2$ fraction (30–50%)	12	305	99	238	2.4
After Sephadex treatment	52	108	240	700	2.9
After DEAE-cellulose column[b]	5	10	690	1560	2.3
Final preparation	4	2.2	2100	4600	2.2

[a] Micromoles of SO_2 or CO_2 formed per mg of protein per hour. [b] Individual component fractions exhibited ratios (B/A) of from 2.1 to 2.6.

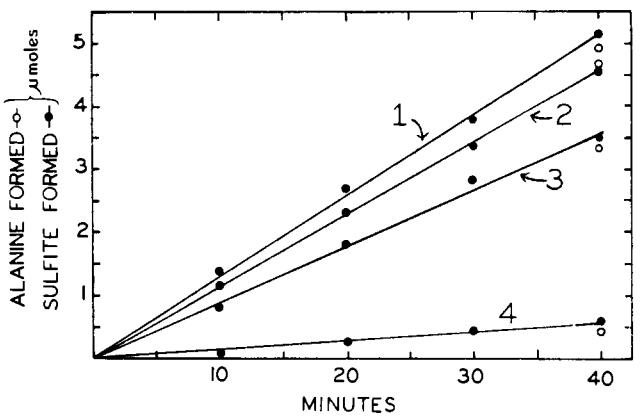


FIG. 1.—Effect of pyridoxal 5′-phosphate and α-ketoglutarate on the desulfination of L-cysteine sulfinic acid. The reaction mixtures contained 3.6 μg of enzyme and other components as described under Methods. Curve 1, cysteine sulfinate, α-ketoglutarate, and pyridoxal phosphate; curve 2, cysteine sulfinate and α-ketoglutarate; curve 3, cysteine sulfinate and pyridoxal phosphate; curve 4, cysteine sulfinate. The values for alanine formation are indicated by open symbols.

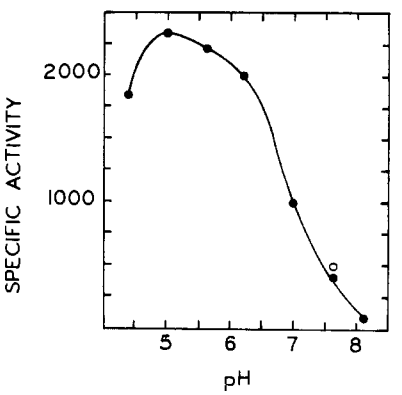


FIG. 2.—Effect of *p*H on the desulfination of cysteine sulfinic acid. The reaction mixtures contained L-cysteine sulfinic acid (30 μmoles), pyridoxal phosphate (0.5 μmole), α-ketoglutarate (0.5 μmole), buffer (60 μmoles), and enzyme (2.24 μg), in a final volume of 1.5 ml. Sodium acetate buffer was employed at *p*H 4.4, 5.0, 5.6, and 6.2; Veronal buffer was employed at *p*H 7.0, 7.6, and 8.2. Potassium phosphate (open symbol) was also used at *p*H 7.6.

lar spots containing known amounts of standard alanine were eluted and treated with ninhydrin according to the procedure of Giri *et al.* (1952a,b). Other solvents used for the paper-chromatographic identification of alanine included ethanol-water (78:22) and methanol-water-pyridine (20:5:1).

RESULTS

Purification of the Activities.—As indicated in Table I, the ratio of aspartate β-decarboxylase activity to cysteine sulfinate desulfinase activity remained the same, within experimental error, during the several steps used in purification. Under the conditions employed for the determination of enzymatic activity, the ratio of decarboxylase to desulfinase activities was approximately 2. The relative decarboxylase and desulfinase activities of the enzyme preparation previously described (Novogrodsky *et al.*, 1964) were the same as those obtained with the present preparation of enzyme.

Effect of Pyridoxal Phosphate and α-Ketoglutarate on the Desulfination of Cysteine Sulfinate.—The desulfination of cysteine sulfinate was markedly activated by both pyridoxal 5′-phosphate and α-ketoglutarate in a manner similar to that observed for the decarboxylation of aspartate (Fig. 1) (Meister *et al.*, 1951; Novogrodsky and Meister, 1964). Similar activation was observed when pyruvate was used in place of α-ketoglutarate. The formation of sulfite was accompanied by equimolar formation of alanine. Alanine was identified by paper chromatography in several solvent systems and also by gas-liquid chromatography[1] (Johnson *et al.*, 1961). The *p*H optimum for the desulfination reaction was approximately 5 and therefore the same as observed previously for the decarboxylation of aspartate (Fig. 2).

Effect of Preincubation of the Enzyme with Substrates.—As indicated in Table II, preincubation of the enzyme with aspartate led to inactivation of cysteine sulfinate desulfinase activity. Inactivation was markedly reduced when the enzyme was preincubated with both aspartate and α-ketoglutarate. Analogous experiments were carried out in which the enzyme was preincubated with cysteine sulfinate. Aspartate decarboxylase activity was completely inhibited when the enzyme was preincubated with cysteine sulfinate, but after preincubation with cysteine sulfinate and α-ketoglutarate most of the initial aspartate decarboxylase activity remained. Enzyme inactivated by preincubation with either aspartate or cysteine sulfinate was activated by subsequent addition of pyridoxal phosphate.

Ratio of Desulfinase and Transaminase Activities.—Previous studies showed that the enzyme catalyzes several transamination reactions, and that the ratio of the aspartate decarboxylase and α-ketoglutarate-aspartate transaminase activities is 500–600 at *p*H 5 (Novogrodsky and Meister, 1964). The transaminase activity may be conveniently followed by determination of the rate of conversion of α-[5-^{14}C]ketoglutarate to

[1] The authors thank Mr. Peter Polgar of this Department for performing these studies.

TABLE II
EFFECT OF PREINCUBATION OF ENZYME WITH SUBSTRATES

Expt	Preincubation Conditions	Activity: Desulfinase (μmoles SO_2 per 15 min)	Activity: Decarboxylase (μmoles CO_2 per 15 min)
1[a]	Enzyme	0.545 (0.526)	
	Enzyme + aspartate	<0.010 (0.376)	
	Enzyme + aspartate + α-ketoglutarate	0.413 (0.413)	
2[b]	Enzyme		1.55 (1.48)
	Enzyme + cysteine sulfinate		<0.10 (1.59)
	Enzyme + cysteine sulfinate + α-ketoglutarate		1.32

[a] Expt 1: The enzyme (11.2 μg) was preincubated for 15 minutes at 37° in a volume of 1.0 ml containing sodium acetate buffer (900 μmoles, *p*H 5) and, as indicated, L-aspartate (8 μmoles) and sodium α-ketoglutarate (10 μmoles). Desulfinase activity was then determined on 0.2-ml aliquots in a reaction mixture consisting of L-cysteine sulfinate (5 μmoles), sodium α-ketoglutarate (0.5 μmole), and sodium acetate buffer (*p*H 5.0, 140 μmoles), in a final volume of 1.5 ml. Values obtained when pyridoxal 5'-phosphate (0.5 μmole) was also added in the assay are given in parentheses. [b] Expt 2: The conditions were the same as in Expt 1 except that preincubation was carried out with 14 μg of enzyme and L-cysteine sulfinate; aspartate decarboxylase activity was then determined on 0.2-ml aliquots in reaction mixtures consisting of L-aspartate (15 μmoles), sodium α-ketoglutarate (0.5 μmole), and sodium acetate (*p*H 5, 140 μmoles) in a final volume of 1.5 ml.

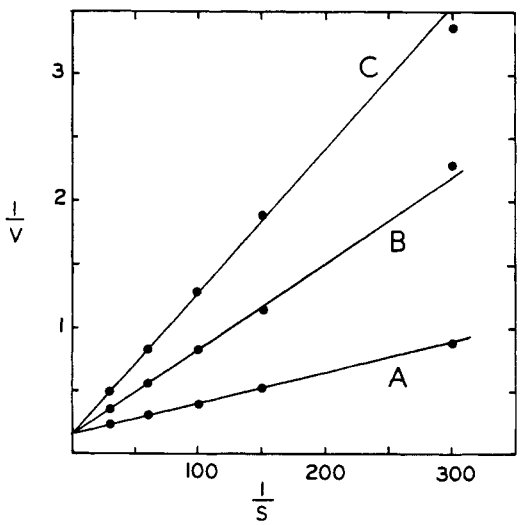


FIG. 3.—Inhibition of desulfination by aspartate. Desulfination was determined as described under Methods with both pyridoxal phosphate and α-ketoglutarate. The concentration of substrate was varied from 0.0033 to 0.033 M. The concentration of L-aspartate was zero (curve A), 0.000833 M (curve B), and 0.00167 M (curve C). The value of V is expressed as specific activity $\times 10^{-3}$.

[^{14}C]glutamate. Experiments carried out with cysteine sulfinate and labeled α-ketoglutarate indicated the formation of significant quantities of glutamate. As shown in Table III, the ratio of desulfination to transamination was, within experimental error, the same as that of aspartate decarboxylation to transamination.

Inhibition of Desulfinase Activity by Aspartate.—Desulfination of L-cysteine sulfinic acid was inhibited by relatively low concentrations of L-aspartate. Treatment of the data according to the method of Lineweaver and Burk (1934) showed that the inhibition was competitive (Fig. 3). The K_i value for aspartate was 4.9×10^{-4} under the conditions described in Figure 3 The K_m value for L-cysteine sulfinic acid was 1.7×10^{-2} M. Although the enzyme exhibited greater affinity for aspartate than for cysteine sulfinate, the extrapolated maximum value for the rate of desulfination (6600 μmoles/mg enzyme per hour) was somewhat greater than the maximum rate of aspartate decarboxylation (4600 μmoles/mg per hour).

Other Studies.—The rate of desulfination was decreased by about 50% when the concentration of acetate buffer was increased from 0.17 to 0.5 M. A similar but less marked effect of acetate concentration on aspartate decarboxylation was observed previously (Novogrodsky and Meister, 1964). Phthalate was found to be a competitive inhibitor of desulfination (K_i, 1.9×10^{-3} M). Phthalate also inhibited aspartate decarboxylation; neither isophthalate nor terephthalate inhibited at a concentration of 0.025 M. Dipicolinic acid and quinolinic acid at a concentration of 0.013 M inhibited desulfination about 80%, while much less inhibition was observed with pyridine 2,5-dicarboxylic acid (3%), pyridine 3,4-dicarboxylic acid (40%), and pyridine 3,5-dicarboxylic acid (30%). Neither activation nor inhibition was observed with 0.013 M $MgCl_2$ or $CaCl_2$.

TABLE III
RATIO OF DESULFINASE AND TRANSAMINASE ACTIVITIES[a]

Incubation Period (min)	Desulfination (A) (SO_2) (μmoles)	Transamination (B) (Glutamate) (mμmoles)	Ratio A/B
20	1.89	3.23	585
40	3.30	5.16	640

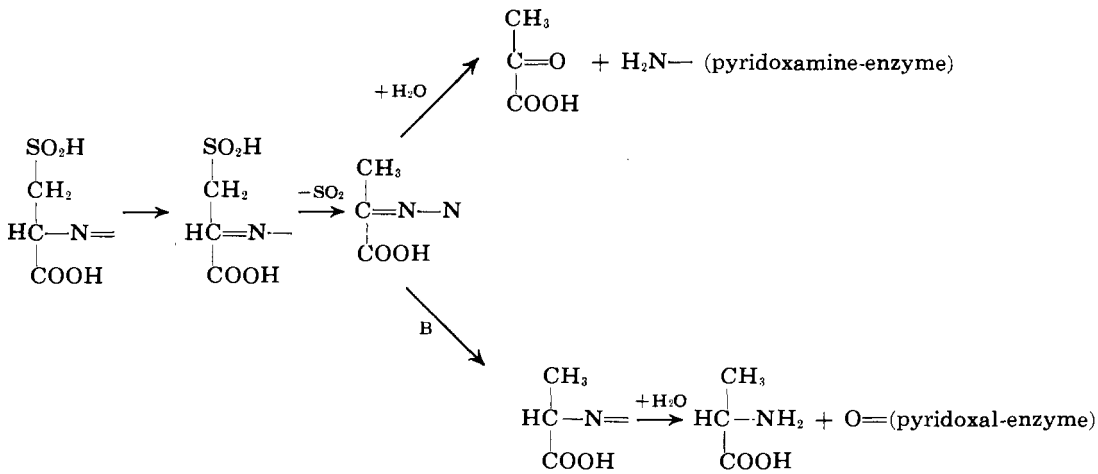
[a] The conditions were the same as described in Fig. 1 (curve 2) except that 3 μg of enzyme and 0.428 μmole of α-[5-^{14}C]ketoglutarate (1.32×10^6 cpm) were used. The formation of [^{14}C]glutamate was determined as previously described (Novogrodsky and Meister, 1964). Under these conditions, but substituting L-aspartate for L-cysteine sulfinate, the ratio of decarboxylation to transamination was 535.

The decarboxylation of aspartate was inhibited about 30% by sodium sulfite (0.0017 M), and such inhibition was reduced when higher concentrations of pyridoxal 5'-phosphate were added. Sulfite reacts readily with pyridoxal phosphate to yield a complex which exhibits an absorbance maximum in the region of 325–330 mμ. Preliminary studies suggest that it is possible to determine the sulfite formed enzymatically from cysteine sulfinate by aspartate decarboxylase or glutamate-aspartate transaminase by following the decrease in absorbance at 388 mμ in the presence of excess pyridoxal phosphate, or the concomitant increase at 325–330 mμ. Such a procedure might provide a very convenient means of determining transaminase activity.

Discussion

A number of investigations have indicated that cysteine sulfinic acid is an intermediate in the metabolism of cysteine and it has been shown that this amino acid undergoes decarboxylation (Bergeret *et al.*, 1952), oxidation, and transamination (Singer and Kearney, 1955). Fromageot and colleagues (1951) reported at first that cysteine sulfinate was desulfinated to alanine by preparations obtained from rabbit liver, but later work by Fromageot's group (Chatagner *et al.*, 1952) and by Singer and Kearney (1955) led these investigators to the conclusion that the observed desulfination was due to transamination of cysteine sulfinate with α-ketoglutarate (or oxaloacetate) to yield β-sulfinylpyruvate, which was postulated to undergo desulfination (possibly nonenzymatically) to yield pyruvate. Although alanine was found after incubation of cysteine sulfinate with preparations of tissues and microorganisms, the formation of this amino acid could readily be explained by transamination between the pyruvate formed from β-sulfinylpyruvate and glutamate (or other amino acids present in the enzyme preparations). This interpretation of the mechanism of desulfination was consistent with the finding of a heat-stable cofactor for the desulfinase reaction in yeast that was replaceable by α-ketoglutaric acid (Chatagner *et al.*, 1952). However, the studies on the β-decarboxylation of aspartate (Meister *et al.*, 1951; Novogrodsky *et al.*, 1963) and the present work indicate that the activation by α-keto acids of the β-decarboxylation reaction and of the analogous desulfination reaction is not associated with transamination of very large amounts of substrate. It is possible that some cysteine sulfinate may be directly desulfinated to alanine. This suggestion is consistent with the recent finding of Sumizu (1961), who obtained evidence for conversion by a rat liver preparation of cysteine sulfinate to unlabeled alanine in the presence of [^{14}C]pyruvate. This reaction required pyridoxal phosphate, but information concerning the effect of pyruvate and other α-keto acids on the reaction was not reported. Despite these considerations it seems probable that the release of sulfite from cysteine sulfinate can occur by the transamination pathway since cysteine sulfinate is an excellent substrate for glutamate-aspartate transaminase. The latter observation was first reported by Singer and Kearney (1955) and has been confirmed in studies in this laboratory with a more purified preparation of the transaminase (obtained from pig heart) than was on hand at the time of the earlier studies. The available data indicate that the formation of sulfite from cysteine sulfinate can take place by at least two mechanisms, but the relative quantitative significance of these in different cells requires further study. The relatively high K_m value for cysteine sulfinate for the present enzyme suggests that the observed desulfination may not be of great physiological importance; on the other hand, this organism does not seem to have appreciable glutamate-aspartate transaminase activity. It would be of interest to learn whether the aspartate β-decarboxylase of *Desulfovibrio desulfuricans* (Cattanéo-Lacombe *et al.*, 1958) also catalyzes desulfination and whether this reaction is of particular significance in the metabolism of this organism. It would also be pertinent to determine whether animal tissues possess an aspartate β-decarboxylase with specificity and other properties similar to those of the *A. faecalis* enzyme.

The ability of the purified aspartate β-decarboxylase to act on cysteine sulfinate is consistent with the demonstrated broad specificity of this enzyme in transamination (Novogrodsky and Meister, 1964). It seems probable that the ketimine form of the cysteine sulfinate–pyridoxal–enzyme complex is at least as susceptible to desulfination as free β-sulfinylpyruvate. However, the formation of the latter compound has not yet been demonstrated, and the possibility exists that the desulfination of cysteine sulfinate catalyzed by glutamate-aspartate transaminase as well as that catalyzed by aspartate β-decarboxylase involve loss of sulfite from the same type of Schiff-base intermediate.



In pathway A (transaminase-catalyzed) the pyridoxamine form of the enzyme is produced, while the pyridoxal enzyme is formed in pathway B. As in the reaction with aspartate, the enzyme that catalyzes desulfination by pathway B occasionally catalyzes a reaction of type A, thus accounting for inactivation by substrate.

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Studies of Phosphorus Metabolism by Isolated Nuclei. II. Investigation of Optimal Conditions for Its Demonstration*

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Rat liver nuclei isolated by sedimentation in dense sucrose have been found capable of converting inorganic orthophosphate to forms not extractable by organic solvents such as phosphomolybdate. The phosphorylation proceeds linearly with time and is maximal at *p*H 7.0 and 30°. The process is aerobic; is relatively insensitive to dinitrophenol, sodium cyanide, or antimycin A; requires Mg^{2+}; and is stimulated by fluoride. Label is incorporated into both the acid-soluble and acid-insoluble fractions produced by perchloric acid treatment of the nuclei. Mitochondria isolated in similar fashion in dense sucrose retain their normal phosphorylative activity. The evidence indicates that liver nuclei possess inherent phosphorylative activity which is unique to the nucleus itself.

The use of labeled precursors has amply shown that nuclei can synthesize proteins and nucleic acids (Allfrey, 1954; Allfrey *et al.*, 1957). Other studies have shown that isolated nuclei of calf and rat thymus can convert endogenous nucleoside mono- and diphosphates to nucleoside triphosphates upon aerobic incubation (Osawa *et al.*, 1957; Creasey and Stocken, 1959). Such observations indicate that nuclei possess an active phosphorylation system, akin in many ways to that of mitochondria. Recently it has become increasingly clear that cell nuclei have extensive enzyme complements for possible energy transduction (McEwen *et al.*, 1963; Siebert, 1963). The systems responsible for phosphorylation by calf thymus nuclei have been studied in detail (McEwen *et al.*, 1963). Phosphorylation by thymus nuclei is principally aerobic and may be inhibited by DNP, NaCN, and the like, but it is unaffected by 95% CO–5% O_2, 3 mM Ca^{2+}, or methylene blue. The latter properties are felt to indicate that the phosphorylation is truly a nuclear process.

While the properties of thymus nuclear phosphorylation have been clarified extensively, the possibility that nuclei of radioinsensitive tissues such as liver or kidney possess such an activity is not so clear-cut. Isolated nuclei of rat liver and kidney can incorporate amino acids into protein and convert P_i to covalent form (Rees and Rowland, 1961; Rees *et al.*, 1962); and it has been postulated that such nuclei possess a phosphorylation system. However, the difficulty in studying nuclei of such tissues lies in that, upon exposure to aqueous-sucrose media, the nuclei tend to lose all or most of their endogenous complement of nucleotides and some protein (McEwen *et al.*, 1963). In addition, it is impossible to isolate liver nuclei in pure state by straightforward repetitive sedimentation in 0.25 M sucrose systems (Roodyn, 1962; Rees and Rowland, 1961). This difficulty may be obviated if a sedimentation in dense sucrose is employed to separate by flotation possible subcellular contaminants of lower density than the nuclei. Liver nuclei so isolated have been found capable of incorporating amino acids into protein (Rendi, 1960).

In early experiments on rat spleen and liver nuclei (Penniall *et al.*, 1962) in which we sought to extend the work of Creasey and Stocken (1959), we found that liver nuclei isolated by means of 2.2 M sucrose have a capacity to convert $^{32}P_i$ to covalent form. Our results verified reports that liver nuclei lost most of their complement of nucleotides upon isolation.[1] However, despite this objectionable aspect to the isolation of liver nuclei in aqueous media, it was felt significant that they retained some phosphorylative activity. Therefore we have explored the problem further, since such a system affords a chance to study liver nuclei under conditions wherein extranuclear contamination is truly minimal or absent. In this paper we wish to present the results of such studies. The first paper in this series has appeared in print (Penniall *et al.*, 1963).

Experimental Procedures

Isolation of Nuclei.—In this work mature male rats of Sprague-Dawley descent were used. Upon ether anesthesia of the rats the livers were perfused with cold isotonic saline prior to excision. In all manipulations after perfusion the tissues or extracts therefrom were kept at 0–3°. Upon excision each liver was homogenized singly in a medium containing 0.25 M sucrose: 3mM $MgCl_2$–5 mM triethanolamine, *p*H 7.0, in a Teflon-glass Potter-Elvehjem apparatus, and the pooled homogenates were well mixed with further medium to give a final ratio of 60 ml of medium/liver. This homogenate was filtered with gentle suction through two layers of fine-weave nylon cloth and centrifuged 15 minutes at 900 × *g* in the International

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[1] R. Penniall, unpublished data.