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A Rat Liver System that Catalyses a Pyridoxal Phosphate-Independent αβ-Elimination

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1. Attempts were made to demonstrate the presence of pyridoxal phosphate in the rat liver system catalysing the αβ-elimination of L-serine *O*-sulphate. 2. Methods designed to resolve protein-bound cofactor, spectroscopic examination of a purified enzyme system and attempted reactivation of apo-(alanine aminotransferase) failed to demonstrate the presence of pyridoxal phosphate. 3. The activity of the αβ-eliminating system remained constant in vitamin B_6-deficient animals even though the activities of other pyridoxal phosphate-dependent systems fell markedly. 4. The metabolism of L-serine *O*[^{35}S]-sulphate *in vivo* appears to be normal in vitamin B_6-deficient animals. 5. No incorporation of tritium into the αβ-eliminating system occurred after administration of tritiated pyridoxine to experimental animals.

A rat liver system catalysing the αβ-elimination of L-serine *O*-sulphate has been shown thus far to have a limited specificity, being active only towards the L-serine *O*-sulphate residue and β-chloroalanine (Thomas & Tudball, 1967). Previous reports on the dehydrochlorination of β-chloroalanine *in vitro* have variously claimed that the process is catalysed by homoserine dehydratase (Gregerman & Christensen, 1956) and by either cystathionine synthetase or L-serine dehydratase (Nagabhushanam & Greenberg, 1965), activities known to be absent from the present system under investigation. The exact significance of the observations of Nagabhushanam & Greenberg (1965) is, however, doubtful at present. These authors claimed that cystathionine synthetase and L-serine dehydratase activities were properties of a single protein, though more recent investigations (Brown, Mallady & Roszell, 1966; Nakagawa & Kimura, 1968) have demonstrated the separate identity of these activities.

Significantly, perhaps, all enzyme systems known to catalyse αβ-eliminations require the participation of pyridoxal phosphate as an obligatory cofactor. It is noteworthy that the αβ-elimination of L-serine *O*-sulphate may be conveniently brought about *in vitro* by pyridoxal phosphate and metal ions (Thomas, Dodgson & Tudball, 1968), a similar reaction having been reported for β-chloroalanine by Gregerman & Christensen (1956). No conclusive evidence has yet been presented on the participation of pyridoxal phosphate in the enzyme-catalysed degradation of L-serine *O*-sulphate, although several well-known carbonyl reagents had variable effects on enzyme activity (Thomas & Tudball, 1967). The present investigation was undertaken to demonstrate conclusively whether or not pyridoxal phosphate participates in the enzyme-catalysed degradation of L-serine *O*-sulphate.

A preliminary account of this work has already been published (Thomas, Tudball & Fowler, 1968).

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MATERIALS AND METHODS

Chemicals. The potassium salts of ^{35}S-labelled and unlabelled L-serine *O*-sulphate were prepared as described by Tudball (1962). Pyridoxine hydrochloride was purchased from Sigma (London) Chemical Co., London S.W.6. Isonicotinic acid hydrazide (isoniazid) was a preparation from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Tritiated pyridoxine and $H_2{}^{35}SO_4$ were obtained from The Radiochemical Centre, Amersham, Bucks. Triton X-100 was obtained from British Drug Houses Ltd., Poole, Dorset, and 2,5-diphenyloxazole from Beckman Instruments Inc., Fullerton, Calif., U.S.A.

Normal and vitamin B_6-deficient diets were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Both the normal and vitamin-deficient diets were admixed with a vitamin fortification mixture free of pyridoxine hydrochloride (Nutritional Biochemicals Corp.) for feeding purposes.

Experimental animals. M.R.C. hooded rats were used throughout.

Assay of enzyme activities. *O*-Phospho-L-serine phosphatase (phosphoserine phosphohydrolase, EC 3.1.3.3) activity was assayed by the procedure of Borkenhagen & Kennedy (1959). Alanine aminotransferase (L-alanine–2-oxoglutarate aminotransferase, EC 2.6.1.2) activity was measured by the method of Tonhazy, White & Umbreit (1950). L-Serine dehydratase [L-serine hydro-lyase (deaminating), EC 4.2.1.13] activity was measured by the method of Selim & Greenberg (1959). L-Serine *O*-sulphate-degrading activity was assayed by the procedure of Thomas & Tudball (1967).

Preparation of purified enzyme. The stage 7 preparation described by Thomas & Tudball (1967) was employed as the starting material. CM-Sephadex was allowed to swell in a large excess of 0·05M-sodium acetate–acetic acid buffer, pH 6·0 (containing a final concentration of 5·0mM-2-mercaptoethanol), for 24hr. A column (2cm. × 10cm.) was prepared and allowed to equilibrate with the same buffer. The stage 7 preparation (20ml. of solution containing 19mg. of protein) was dialysed against two changes of the same buffer for 12hr. (total volume 16l.) and then applied to the column. Elution of L-serine *O*-sulphate-degrading activity was achieved by washing the column with the above buffer at a flow rate of 14ml./hr. The eluate was collected in 2·5ml. portions, fractions 5–7 containing the bulk of the activity. These fractions were pooled and dialysed against 0·1M-tris–HCl buffer, pH 7·0 (containing a final concentration of 5·0mM-mercaptoethanol). The final preparation (7·5ml. of solution containing 6·5mg. of protein) represented a yield of 2·6% of total activity with a corresponding 520-fold purification.

EXPERIMENTAL AND RESULTS

Spectroscopic investigations with the purified enzyme system. Spectroscopic examination of the purified enzyme preparation between 220 and 650nm. did not reveal an absorption typical of that provided by well-documented pyridoxal phosphate-containing systems (Dupourque, Newton & Snell, 1966; Labow & Robinson, 1966; Martinez-Carrion & Jenkins, 1963). If pyridoxal phosphate was present, then it should be possible to induce an alteration in the spectroscopic pattern of the system. The presence of pyridoxal phosphate in enzymes has been demonstrated by changes in spectral properties after addition of substrate (Dupourque *et al.* 1966), adjustment of pH of medium (Jenkins & Sizer, 1959; Shukuya & Schwert, 1960) and addition of carbonyl reagents (Matsuo & Greenberg, 1958; Yasanobu & Yamada, 1963). When similar experiments were performed in the present investigation, no evidence of the required spectral changes was obtained.

Attempted resolution of the enzyme. The involvement of pyridoxal phosphate in a system may only be unequivocally demonstrated by the production of an inactive apoenzyme that may be subsequently reactivated after the addition of cofactor. A number of authors have described methods for the resolution of pyridoxal phosphate from a holoenzyme, the procedure being dependent on the firmness of binding of the cofactor. The methods described by Blakley (1955), Cori & Illingworth (1957), Matsuo & Greenberg (1959), Selim & Greenberg (1959), Braunstein (1960), Nishimura & Greenberg (1961) and Scardi, Scotto, Iaccarino & Scarano (1963) have all been employed without any evidence being provided for the involvement of pyridoxal phosphate.

It is not uncommon, however, for difficulties to be experienced in the resolution of pyridoxal phosphate, since the cofactor appears to be more firmly bound in some systems than in others (Blaschko & Buffoni, 1965). Meister (1965) has suggested that pyridoxal phosphate may be bound quite firmly by phosphodiester and other covalent bonds, as well as by the more common aldimine linkages and ionic bonds found in most of the examples. Such a situation may thus be responsible for the difficulties attending the resolution of pyridoxal phosphate in the present investigation. The well-documented evidence (Brin, Olson & Stare, 1954; Caldwell & McHenry, 1953; Stielau, Freedland & Meyer, 1965) that vitamin B_6 deficiency leads to a decrease in the activities of many well-known enzyme systems requiring pyridoxal phosphate as an obligatory cofactor prompted an investigation into the effects of vitamin B_6 deficiency in rats on the activity of the hepatic system degrading L-serine *O*-sulphate. Vitamin-deficient animals were produced either by feeding them on vitamin-deficient diets, or alternatively by oral administration of isonicotinic acid hydrazide (isoniazid) (Meister & Downey, 1956).

Production of vitamin B_6-deficient rats by employing vitamin-deficient diets. Newly weaned male and female M.R.C. hooded rats (initially averaging 92g. body wt.) were employed. Eighteen pairs of rats were taken for feeding experiments, each pair being litter mates of the same sex and of approximately the same weight. One rat of each pair was then placed on the vitamin B_6-free diet, the other (control) being maintained on the same diet but in addition receiving 100μg. of pyridoxine hydrochloride/day in the drinking water. Animals in each pair were allowed exactly the same food intake in terms of dry weight of feeding material. Initial experiments indicated a lower overall growth rate in animals maintained under these feeding conditions when compared with rats allowed to feed *ad libitum* on a normal diet over a period of 70 days. The lower rate of growth with the animals maintained under the above regimen is principally due to the loss of appetite that accompanies vitamin B_6 deficiency. After 70 days on the vitamin-deficient diet animals developed moist snouts and showed signs of tremor, symptoms normally

associated with severe vitamin B_6 deficiency, but did not exhibit signs of acrodynia.

Enzyme activities of normal and vitamin B_6-deficient animals. Four pairs of rats were killed after 0, 22, 38 and 51 days on the experimental diet. Livers were removed, finely diced and then suspended with the aid of a glass homogenizer in 1·2% (w/v) potassium chloride (1·0mM with respect to EDTA) to give a 10% (w/v) tissue suspension. This suspension was then employed as enzyme source for the assay of alanine aminotransferase, L-serine dehydratase, *O*-phosphoserine phosphatase and L-serine *O*-sulphate-degrading activities. The results are presented in Table 1.

The relationship between pyridoxal phosphate and the aminotransferases is now well established and numerous publications have appeared in which tissue aminotransferase activity is depressed in vitamin B_6-deficient animals (Stielau *et al.* 1965). The activities of hepatic alanine aminotransferase fall dramatically in vitamin B_6-deficient animals to a value that remains unaltered by the addition of pyridoxal phosphate (final concn. 75μM) to the incubation medium. It is known (Radhakrishnamurty & Sabry, 1968) that alanine aminotransferase activity of mitochondria remains unaltered in vitamin B_6 deficiency, whereas the activity of the cell-sap system falls markedly. In the present experiment the results reflect total hepatic aminotransferase activity and are employed solely as a demonstration of vitamin B_6 deficiency.

The L-serine dehydratase activity was also lowered in vitamin B_6 deficiency. Addition of pyridoxal phosphate (final concn. 75μM) to both control and vitamin B_6-deficient assay mixtures resulted in an increase in activity in both cases, although complete reactivation of enzyme activity from vitamin B_6-deficient animals was not achieved, possibly reflecting loss of apoenzyme as a result of vitamin deficiency. This loss of activity may simply reflect the instability of apoenzyme, caused by decreased availability of coenzyme; alternatively there are indications that in some instances apoenzyme synthesis may be induced and regulated by pyridoxal phosphate (Wiss & Weber, 1964).

No significant change was observed in either *O*-phosphoserine phosphatase or L-serine *O*-sulphate-degrading activities. Even though these results indicate that pyridoxal phosphate was unlikely to be involved in the enzymic degradation of L-serine *O*-sulphate, this possibility could not with any certainty be excluded. It was conceivable that the affinity of the system for pyridoxal phosphate was higher than that of most other enzyme systems investigated and that, even under the extreme conditions of vitamin B_6 deprivation employed, sufficient coenzyme remained for maximal activity.

Table 1. *Effects of feeding with vitamin B_6-deficient diets on the activities of hepatic enzymes in the rat*

Results are quoted as average values obtained with four animals, with ranges in parentheses. Experimental procedures are given in the text.

Enzyme system	Experimental conditions	Mean specific activity (units/mg. of protein)			
	Duration of experimental period (days) ...	0	22	38	51
Alanine aminotransferase	Animals receiving pyridoxine-supplemented diet	12·81 (10·18–15·44)	14·37 (9·92–18·82)	12·67 (7·92–17·42)	11·55 (7·12–15·98)
	Animals receiving vitamin B_6-free diet	—	6·30 (3·70–8·90)	5·77 (3·89–7·65)	1·94 (1·23–2·65)
L-Serine dehydratase	Animals receiving pyridoxine-supplemented diet	0·20 (0·17–0·22)	0·17 (0·17–0·18)	0·23 (0·22–0·25)	0·17 (0·16–0·17)
	Animals receiving vitamin B_6-free diet	—	0·01 (0·01–0·02)	0·04 (0·02–0·05)	0·02 (0·01–0·03)
O-Phosphoserine phosphatase	Animals receiving pyridoxine-supplemented diet	0·22 (0·19–0·25)	0·25 (0·75–0·26)	0·20 (0·19–0·20)	0·21 (0·20–0·21)
	Animals receiving vitamin B_6-free diet	—	0·26 (0·26–0·27)	0·22 (0·21–0·23)	0·22 (0·21–0·23)
L-Serine *O*-sulphate-degrading activity	Animals receiving pyridoxine-supplemented diet	1·15 (1·03–1·59)	1·31 (1·03–1·59)	1·05 (0·90–1·20)	1·09 (0·99–1·19)
	Animals receiving vitamin B_6-free diet	—	1·27 (0·99–1·55)	1·18 (1·12–1·24)	1·22 (1·10–1·34)

Table 2. *Effects of oral administration of isoniazid on activities of hepatic enzymes in the rat*

Methods for the assay of enzyme activities and experimental procedures employed are given in the text. Results are quoted as average values, with ranges in parentheses.

Experimental conditions	No. of experimental animals	Mean specific activity (units/mg. of protein)	
		L-Serine *O*-sulphate-degrading enzyme	Alanine aminotransferase
Animals receiving normal diet	10	0·76 (0·72–0·80)	13·52 (10·32–16·72)
Animals receiving normal diet + isoniazid	8	0·80 (0·74–0·86)	3·07 (2·45–5·69)
Animals receiving vitamin B_6-free diet, supplemented with pyridoxine	10	0·72 (0·67–0·77)	13·97 (10·39–17·55)
Animals receiving unsupplemented vitamin B_6-free diet	10	0·78 (0·73–0·83)	8·86 (6·61–11·11)
Animals receiving unsupplemented vitamin B_6-free diet + isoniazid	6	0·68 (0·65–0·71)	3·37 (2·91–3·83)

Production of vitamin B_6-deficient animals by oral administration of isoniazid. It is not always possible to alter the activities of pyridoxal phosphate-dependent systems by employing vitamin B_6-deficient diets for the production of vitamin B_6-deficient animals. However, in some cases, when animals are treated with isoniazid, a well-known pyridoxal antagonist, it has then proved possible to bring about a decrease in the enzyme activities that were previously unaltered by nutritional experiments involving vitamin B_6-deficient diets (see Meister & Downey, 1956). Isoniazid was thus administered to rats before an examination of the hepatic activity of the L-serine *O*-sulphate-degrading system.

The test animals (ten rats, average body wt. 144g.) were fed on a normal stock diet and allowed water *ad libitum*. Each day animals received 480mg. of isoniazid/kg. body wt. in 1ml. of water by gastric tube. The control animals were subjected to identical experimental conditions except that isoniazid was replaced by water (1ml.). In a second experiment ten test animals, maintained on the pyridoxine-deficient diet, were allowed water *ad libitum* but received 300mg. of isoniazid/kg. body wt. Two sets of control animals were employed. In one set ten rats were fed on the pyridoxine-deficient diet, but the drinking water was supplemented with pyridoxine hydrochloride as described above, whereas the other set of control animals received the pyridoxine-deficient diet without pyridoxine added to the drinking water. Both sets of controls received 1ml. of water by gastric tube in place of isoniazid. Experiments were allowed to proceed for 11 days, after which time the animals were killed and their livers examined for alanine aminotransferase and L-serine *O*-sulphate-degrading activities as described above. The results are presented in Table 2.

Again no significant alteration was observed in the L-serine *O*-sulphate-degrading activity, even though acute pyridoxine deficiency had been induced, as exemplified by the fall in alanine aminotransferase activity.

Metabolism of L-serine O-sulphate in vitamin B_6-deficient rats in vivo. A substantial body of information now exists on the metabolism of the L-serine *O*-sulphate residue *in vivo* in the rat (see Tudball, Noda & Dodgson, 1965). An investigation was thus conducted into the fate of the ^{35}S-labelled sulphate ester in vitamin B_6-deficient animals, on the premise that, if pyridoxal phosphate were involved in the enzymic degradation of the ester, then this might manifest itself in a diminished ability on the part of the experimental animal to metabolize the substrate.

Pair-fed test and control animals (three pairs of male rats and three pairs of female rats) were subjected to the feeding regimen described above for the vitamin B_6-deficient-diet experiments for a period of 61 days. At the end of this period 5μmoles of ^{35}S-labelled L-serine *O*-sulphate (specific radioactivity 5μc/mg.) were administered to each animal by intraperitoneal injection. Animals were then housed individually in metabolism cages designed to permit separate collection of urine and faeces. The animals were allowed to feed for only 30min. in 24hr., but were allowed water without restriction, the control animals again receiving water supplemented with pyridoxine hydrochloride as described above. Total urine and faeces samples were collected at intervals of 24hr. and 48hr. after injection, after which time the animals were killed and the ^{35}S content of urine, faeces and carcass was assayed by using the procedures described by Tudball (1962).

No significant change in the overall metabolism *in vivo* was observed when L-serine *O*[^{35}S]-sulphate was administered to either normal or vitamin B_6-deficient animals (Table 3), results that serve to

Table 3. *Distribution of* ^{35}S *in urine of rats injected with* L-*serine* O[^{35}S]-*sulphate*

Results are the mean values obtained with three animals, with the ranges in parentheses. ^{35}S was precipitated and counted as $Ba^{35}SO_4$. The radioactivity of the dose (5 μmoles) corresponded to $8{\cdot}36 \times 10^3$ c.p.m./μmole when measured after hydrolysis as an infinitely thick plate of $Ba^{35}SO_4$. Experimental details are given in the text. Up to 2% of the administered dose was recovered in faeces and carcass.

Experimental conditions	Collection period (hr.)	Distribution of ^{35}S in urine (% of ^{35}S administered): Inorganic sulphate fraction	Combined inorganic and ester sulphate fraction
Male rats maintained on pyridoxine-supplemented diet	0–24	76·0 (69·0–81·0)	85·1 (79·0–88·2)
	24–48	5·0 (3·4–7·8)	5·5 (3·5–8·3)
Male rats maintained on vitamin B_6-free diet	0–24	77·6 (76·0–82·2)	88·2 (84·0–91·0)
	24–48	6·2 (5·7–6·8)	6·9 (6·5–7·5)
Female rats maintained on pyridoxine-supplemented diet	0–24	88·0 (83·2–92·8)	91·3 (90·1–92·5)
	24–48	4·5 (3·9–5·1)	4·7 (3·8–5·6)
Female rats maintained on vitamin B_6-free diet	0–24	82·1 (79·8–84·4)	89·8 (87·8–91·8)
	24–48	5·2 (3·9–6·5)	5·2 (4·7–5·7)

complement those obtained with the assays of enzyme activity *in vitro*.

Assay of the purified enzyme preparation for pyridoxal phosphate-utilizing apo-(alanine aminotransferase). The method of reactivating an apoenzyme that specifically requires pyridoxal phosphate as a cofactor, by utilizing the pyridoxal phosphate contained in another system, has been employed by Mondovi, Costa, Finazzi-Agro & Rotilio (1967) to demonstrate that pyridoxal 5′-phosphate is present as a prosthetic grouping in pig kidney diamine oxidase. The use of this approach for the quantitative assay of extremely small amounts of cofactor (down to 1ng.) has been described by Goryachenkova (1963). This technique was employed in the present investigation, utilizing the enzyme purified on CM-Sephadex as the sole source of cofactor.

The preparation of the apoenzyme of alanine aminotransferase and the technique employed for its subsequent reactivation was carried out exactly as described by Goryachenkova (1963). Initially a calibration curve for standard amounts of cofactor was constructed by using aqueous solutions of pyridoxal phosphate in the range 1–5ng. Samples (1ml.) of the purified enzyme, containing 1mg. of protein/ml., were taken for the assay of pyridoxal phosphate. No reactivation of the apoenzyme of alanine aminotransferase resulted after pretreatment of the L-serine *O*-sulphate-degrading enzyme with either 0·2M-sodium hydroxide or aq. 7% (w/v) trichloroacetic acid or by maintaining the sample at 100° for 2min. (see Goryachenkova, 1963).

Attempted labelling of enzyme in vivo *by employing tritiated pyridoxine*. The accumulated evidence at this juncture indicated that pyridoxal phosphate is not involved in the enzymic degradation of L-serine *O*-sulphate. To provide further corroborating evidence for this view an attempt was made to label the cofactor *in vivo* by employing tritiated pyridoxine. Ten 3-month-old male rats received by intraperitoneal injection 500μg. of tritiated pyridoxine hydrochloride (147mc/mmole) in two equal doses on the first and fifth day of the experiment. Animals were allowed a normal diet and water without restriction. At the end of 9 days the animals were killed and their livers removed. The L-serine *O*-sulphate-degrading activity was then purified to the CM-Sephadex stage as described above. A control experiment was performed in which a comparable dose of tritiated pyridoxine hydrochloride was added to the first suspension prepared from livers excised from untreated animals and again the purification procedure was carried through to the CM-Sephadex stage. Tritium radioactivities were then measured on samples of fractions obtained at each stage of the purification procedure with the Beckman LS-100 liquid-scintillation counter. Samples (50μl.) were added to a scintillation fluid (10ml.) prepared by mixing 2,5-diphenyloxazole (5g.), toluene (1l.) and Triton X-100 (500ml.). No radioactivity was found to be associated with the protein fraction obtained at the CM-Sephadex stage.

DISCUSSION

From the results obtained in the present investigation it can now be stated with some certainty that the enzyme system of rat liver that catalyses the $\alpha\beta$-elimination of L-serine *O*-sulphate does not require the participation of pyridoxal phosphate as a cofactor. This is probably the first example of a system catalysing such a reaction that does not

involve pyridoxal phosphate as an obligatory cofactor. Some caution is clearly necessary when attempting to interpret the significance of this observation, as it has been demonstrated that L-serine *O*-sulphate may be employed as a 'quasi' substrate for aspartate aminotransferase (John, Fasella, Thomas & Tudball, 1968) and alanine aminotransferase (N. Tudball & R. A. John, unpublished work), both systems that require pyridoxal phosphate. However, the system degrading L-serine *O*-sulphate is clearly distinguishable from either of these activities.

No evidence has yet been provided that L-serine *O*-sulphate residues occur in Nature. If such residues do not occur naturally then a role for the enzyme system other than that involving degradation of L-serine *O*-sulphate must be sought. Extensive searches for the presence of other activities associated with the L-serine *O*-sulphate-degrading activity have thus far met with little success, apart from the observation that β-chloroalanine will also act as a substrate for this system (Thomas & Tudball, 1967). It is noteworthy that β-chloroalanine may also undergo an $\alpha\beta$-elimination reaction under the agency of aspartate aminotransferase and alanine aminotransferase (N. Tudball & R. A. John, unpublished work). Various reports in the past have appeared in which a variety of activities have been implicated in a similar degradation of β-chloroalanine (Gregerman & Christensen, 1956; Nagabhushanam & Greenberg, 1965). In the light of more recent observations, these earlier results clearly deserve clarification. Such investigations may even lead to a broadening in the spectrum of enzymes at present known to utilize both β-chloroalanine and L-serine *O*-sulphate as 'quasi' substrates.

It has been usual to assume that all enzyme systems catalysing $\alpha\beta$-elimination reactions of necessity require the participation of pyridoxal phosphate as an obligatory cofactor. The system at present under investigation thus represents a unique example of an $\alpha\beta$-elimination that does not require pyridoxal phosphate. This observation, coupled with the report that a pyruvoyl group performs the role normally reserved for pyridoxal phosphate in the histidine decarboxylase of *Lactobacillus* 30a (Riley & Snell, 1968), may prelude reports of other activities that are independent of the presence of pyridoxal phosphate, a factor previously thought to be indispensable for a variety of enzyme-catalysed reactions.

Owing to the present lack of information on a possible physiological function for the L-serine *O*-sulphate-degrading system this substrate is still being used in conjunction with studies on the rat liver system. It is hoped, however, by a systematic investigation into the structural requirements of the enzyme, to pinpoint its true physiological function.

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