solution of styrene oxide in order to have 6 final styrene oxide concentrations ranging from 0.1 to 3.0 mM. Blank values due to non-enzymatic opening of the epoxide were subtracted from the 2 highest concentrations. Enzyme activities are expressed in nmoles/min/mg of protein.
Statistics. At least 3 animals were used for each species. For the mouse, 3 different pools of 5 livers each were used. Kinetic parameters were calculated according to the Woolf plot.
Results and discussion. Table 1 reports the apparent K_m and V_{max} values for styrene epoxide forming monooxygenase, and table 2 those for the epoxide hydratase, in the 4 animal species considered. Table 3 shows the apparent K_m and V_{max} ratios of the epoxide hydratase to the monooxygenase in the different species. In the mouse and rabbit, K_m values for the epoxide hydratase are 18.2 and 6.4 times those of the monooxygenase, while the specific activity of the hydratase for the rabbit is only twice that of the monooxygenase and is almost the same in the mouse.
This broad variability of K_m might imply that at the styrene concentrations (0.1–5 mM) we used in vitro, which presumably reflect those in vivo, the affinity of styrene epoxide for the hydratase is the rate limiting step for the overall metabolic transformation of styrene. This means that it is the speed of hydration of styrene epoxide and not of its formation that determine the rate at which styrene is detoxified.
The effect was less pronounced in the rat and guinea-pig. Table 1 indicates the broad inter-species variability in the K_m of styrene monooxygenase, whereas homogeneous values were found for the epoxide hydratase. These findings are in agreement with data that show there are multiple forms of P-450 dependent monooxygenases with different affinities[8,9].
The greater affinity of styrene monooxygenase, compared to the hydratase, and its considerable inter-species variability seems an important factor in establishing which animal species is most suitable as a model for studies on carcinogenesis. The species with higher rates of epoxide formation (mouse and rabbit) and slower epoxide hydration should be preferred for research into toxicity, such as liver necrosis or tumours in target organs due to accumulation of highly reactive epoxidic metabolic intermediates of foreign compounds.

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Coupling of fructose-1,6-P_2 to aminated agarose by Schiff base reduction. Affinity chromatography of yeast aldolase[1]

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Summary. Fructose-1,6-P_2 was immobilized by sodium borohydride reduction of the Schiff base formed with aminated agarose (AH-Sepharose 4B ®). The coupling occurs with high yield (25 μmoles immobilized fructose-1,6-P_2 per ml packed gel) at neutral pH and room temperature. Schiff base reduction thus provides a convenient and mild coupling procedure for sugar phosphates preserving their labile phospho ester bonds. As exemplified by a new isolation procedure for fructose-1,6-P_2 aldolase from yeast, sugar phosphates insolubilized in this manner may be used for affinity chromatography of the corresponding enzymes, provided that contaminating unspecific phosphatases are removed in a preceding fractionation step.

Sugar phosphates might afford suitable ligands for affinity chromatography of a large number of enzymes. However, the majority of sugar phosphates are labile to hydrolysis by, e.g., alkaline or acid pH, at temperatures above room temperature[4,5], the conditions required for the presently available coupling procedures for carbohydrates[6,7]. This report presents a coupling procedure which is operative at room temperature in the neutral pH range and thus does not affect labile phospho ester bonds. Its practicability was tested by isolating aldolase from yeast by affinity chromatography with insolubilized fructose-1,6-P_2.
Materials. Baker's yeast was purchased from Presshefefabrik Hindelbank; DEAE-Cellulose (DE 52) from Whatman; AH-Sepharose 4B (cross-linked agarose in bead form) from Pharmacia Fine Chemicals; sodium borohydride, ethylenediamine tetraacetic acid, 2-mercaptoethanol and 4-nitrophenyl phosphate from Fluka; enzyme grade ammonium sulfate from Schwarz/Mann; phenylmethylsulfonyl fluoride from Sigma. Fructose-1,6-P_2 tetracyclohexylammonium salt (for aldolase activity assay), fructose-1,6-P_2 trisodium salt, NADH, glucosephosphate isomerase (EC 5.3.1.9), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and glycerol-3-phosphate dehydrogenase/triosephosphate isomerase (EC 1.1.1.8/EC 5.3.1.1) were from Boehringer. Alkaline phosphatase (EC 3.1.3.1) was prepared from E. coli[8].
Methods. Aldolase activity was determined by the coupled assay with glycerol-3-phosphate dehydrogenase omitting 2-mercaptoethanol[9]. In the first steps of the preparation, protein concentrations were determined by the biuret method calibrated with bovine serum albumin; concen-

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trations of clear protein solutions were determined spectrophotometrically using $E_{280} = 1.02$ $(mg/ml)^{-1}$ cm^{-1} [10]. The activity of fructose-1,6-bisphosphatase (EC 3.1.3.11) was determined in a coupled assay with glucosephosphate isomerase and glucose-6-phosphate dehydrogenase[11]. Alkaline phosphatase was assayed in 1 M NaCl, 10 mM Tris-Cl, 1 mM 4-nitrophenyl phosphate in a total volume of 3 ml (pH 8.0, 25 °C). The absorption of 4-nitrophenolate formed was measured at 400 nm ($\varepsilon_{400} = 16.8$ mM^{-1} cm^{-1}). The amount of fructose-1,6-P_2 covalently attached to agarose after reduction with $NaBH_4$ was measured by determining inorganic phosphate[12] released from the charged agarose on treatment with alkaline phosphatase (0.8 U/ml) in 50 mM Tris-Cl (pH 8.0) at room temperature for 30 min. Inorganic phosphate thus determined was referred to the dry weight of the water-washed and lyophilized material. Following starch gel electrophoresis (pH 8.6, 15 V/cm, 3 mA/cm², 4 °C, 5 h) with discontinuous buffer system[13], one gel slice was stained for protein with Coomassie G-250[14], the other one for aldolase activity[15]. Polyacrylamide gel electrophoresis was carried out at pH 8.9 with 7.5% acrylamide[16] or at pH 8.3 with 10% acrylamide and 1% sodium dodecylsulfate[17]. Prior to electrophoresis in sodium dodecylsulfate, the samples were boiled for 15 min in 62.5 mM Tris-Cl/10% glycerol/1% sodium dodecylsulfate (pH 6.8).

Isolation of fructose-1,6-P_2 aldolase from 185 g baker's yeast

	Total activity (U)	Yield (%)	Specific activity (U/mg)	Purification factor
1. Supernatant of disrupted cells	30 000	100	1.5	1
2. Heated supernatant after dialysis	20 000	67	3	2
3. Pool after DEAE-cellulose chromatography	12 000	40	50	33
4. Pool after fructose-1,6-P_2 agarose affinity chromatography	3 000	10	100	67

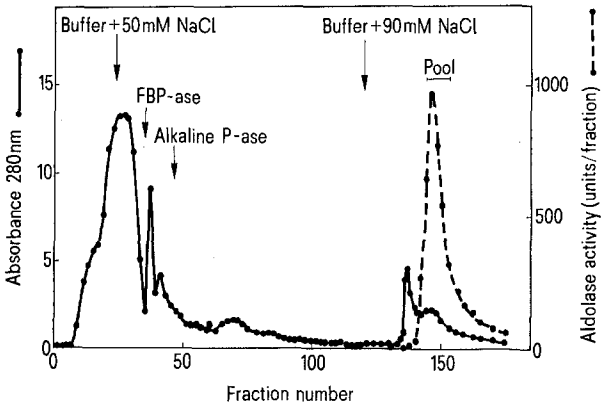

Fig. 1. DEAE-cellulose chromatography of crude yeast extract. Separation of aldolase from fructose-1,6-bisphosphatase (FBP-ase) and alkaline phosphatase (P-ase). The fraction volume was 7.2 ml. For other details see text.

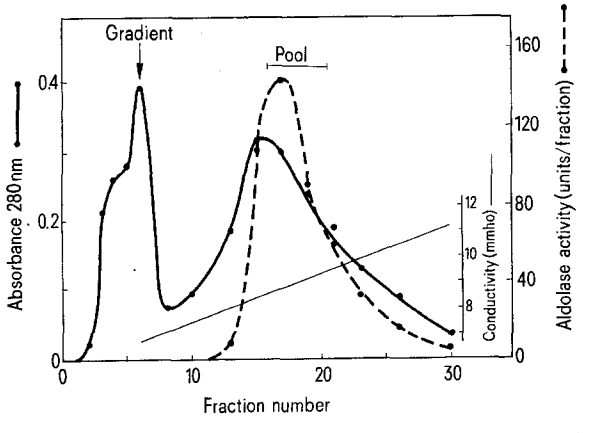

Fig. 2. Affinity chromatography on fructose-1,6-P_2 agarose. The fraction volume was 5 ml. For other details see text.

Coupling of fructose-1,6-P_2 to AH-Sepharose 4B. The commercially available agarose derivative AH-Sepharose 4B contains primary amino groups on 6 carbon long spacer groups. The Schiff base formed between aminated agarose and the carbonyl group of fructose-1,6-P_2, the later added in excess, was reduced with sodium borohydride and thus converted into a stable secondary amine. Detailed Procedure: AH-Sepharose 4B (7.5 g) is swollen in 200 ml 0.5 M NaCl at 4 °C for 12 h and subsequently washed with 1.5 l 0.5 M NaCl and 500 ml 0.5 M sodium acetate/0.1 M NaCl (pH 5.0) on a Büchner funnel. Fructose-1,6-P_2 trisodium salt (100 mM) is added to the AH-Sepharose 4B suspended in 50 ml of the second wash buffer. The suspension is gently rocked at room temperature for 2 h. During this period maximum Schiff base formation is reached. For reduction of the Schiff base linkages, the pH value is adjusted to 7.0 with NaOH and 250 mg sodium borohydride are added in 5 portions in intervals of 1 min, while the pH value is kept constant at 7.0 by the addition of HCl. After washing with 2 l of water, the fructose-1,6-P_2 agarose is stored in 1 M NaCl at pH 5.0 and 4 °C.

By this procedure, approximately 0.1 mmole fructose-1,6-P_2 is covalently attached to 1 g of dry AH-Sepharose 4B. The equivalent of 1 g dry Sepharose being 4 ml sedimented gel, this value corresponds to a concentration of 25 mM of the immobilized ligand in the packed gel. Fructose-1,6-P_2 agarose is stable for at least 3 weeks. Following chromatography it may be regenerated for reuse by 1 M NaCl.

Isolation of fructose-1,6-P_2 aldolase from yeast. Step 1: Baker's yeast (185 g wet weight) is suspended in 100 ml 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride (pH 7.5) at 4 °C. Cells are disrupted in glass flasks (70 ml) filled with equal volumes of yeast suspension and glass beads (1.0–1.5 mm diameter) that are shaken in a shaker machine at 3500 cycles min^{-1} with a lateral displacement of 2.2 cm. The flasks are cooled with CO_2. Cell disruption is complete within 2 min. The homogenate is centrifuged for 30 min at 20,000 × g and 4 °C. The sediment is re-extracted with additional 135 ml buffer.

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Step 2: The 2 supernatants (pH ~ 6) are combined, heated in presence of 5 mM fructose-1,6-P_2 to 48 °C within 2–3 min and after 2 min at this temperature cooled with ice water. The precipitate formed is removed by centrifugation for 60 min at 20,000 × g and 4 °C. The supernatant is dialyzed against 5 l 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride (pH 7.5) at 4 °C for 15 h.

Step 3: The dialyzed supernatant (150 ml, ~ 20,000 U aldolase activity) is chromatographed on a DE 52 column (1.9 × 43 cm). The column is packed under pressure (0.7 kp cm^{-2} = 8.75 psi) and equilibrated with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride (pH 7.5) at 4 °C. Following application of the sample (57 ml/h), the column is washed with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/50 mM NaCl (pH 7.6) until the absorbance at 280 nm of the eluate has decreased to zero. This fractionation step removes both unspecific alkaline phosphatase and fructose-1,6-bisphosphatase from the aldolase activity containing fractions which are subsequently eluted with 50 mM Tris-Cl/0.1 mM EDTA/0.2 mM phenylmethylsulfonyl fluoride/90 mM NaCl (pH 7.6) (figure 1). If not removed, contaminating phospho esterase activity would hydrolyze the immobilized phospho ester ligand used in affinity chromatography.

Step 4: The fractions containing aldolase activity were pooled and subjected to affinity chromatography on a fructose-1,6-P_2 agarose column (0.9 × 6.5 cm) equilibrated with 10 mM Tris-Cl (pH 7.5). The apparent maximum aldolase binding capacity of fructose-1,6-P_2 agarose is 3000 U/g dry weight; after binding of this amount of aldolase activity, the activity concentration of the eluate is the same as that of the solution pumped onto the column (~ 50 U/ml). In order to obtain satisfactory purification, the amount of aldolase applied to the column should not exceed $^1/_3$ of the maximum capacity, i.e., 1000 U per run on the present 4 ml-column. For elution a linear phosphate gradient from 10 mM Tris-Cl/90 mM NaCl (pH 7.6) to 10 mM Tris-Cl/90 mM NaCl/100 mM sodium phosphate (pH 7.6), total volume 2 × 150 ml, was applied (figure 2). Elution with fructose-1,6-P_2 instead of inorganic phosphate did not improve the separation effect. The retardation of aldolase appears to be due to a specific interaction with the immobilized phospho ester ligand. Retardation by ion exchange effects seems to be excluded in view of the isoelectric point of yeast aldolase at pH 5.5[10]. AH-Sepharose 4B without linked fructose-1,6-P_2 did not retard aldolase and brought no purification of the applied enzyme solution. A summary of the purification procedure is given in the table. The yield given for the affinity chromatography is the sum of individual runs with fractional quantities of the enzyme preparation. Amino acid analyses of the purified aldolase closely corresponded with previously reported data[10]. The enzyme proved homogeneous on starch gel electrophoresis stained for protein and enzymatic activity and on polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate showed several lighter fragments, possibly indicative of a sodium dodecyl sulfate activated protease as observed in the purification of another yeast enzyme[18]. The purified aldolase when stored at –20 °C has, after 1 month, still 90% and, after 3 months, 50% of its initial activity. Addition of 100 mM 2-mercaptoethanol or 0.2 mM phenylmethylsulfonyl fluoride or storage as a suspension in 90% saturated ammonium sulfate does not increase stability.

In conclusion, reduction of the Schiff base formed with aminated agarose is a useful procedure for immobilizing fructose-1,6-P_2 and possibly other glycolytic substrates. Affinity chromatography of a prepurified yeast extract yielded an aldolase preparation comparable to that obtained by other procedures[9]. The non-coincidence of the protein and activity concentration curves in the elution profile (figure 2) appears to indicate, however, a possibly general limitation of the application of sugar phosphates as ligands in affinity chromatography, viz. ion exchange effects and/or unspecific retardation of other enzymes operating on phopho ester substrates.

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Action of iodine on the tomato pectinesterase

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Summary. The organophosphates and carbamates did not inhibit the isolated tomato pectinesterase. Therefore this enzyme cannot be defined as serine-type esterase. The enzyme is inhibited by iodine and the inhibition (irreversible and non-competitive) is dependent on the degree of enzyme purification.

The previous papers[1,2] described a resistance of pectinesterase (pectin pectyl-hydrolase, EC 3.1.1.11) against chemical agents such as iodine, cyanide, formaldehyde and others. In these works, crude preparations of tomato and microbial pectinesterase were used. From the esterase-inhibitors of DIP-type, the use of diisopropylphosphorfluoridate on microbial pectinesterase had a negligible effect[3]. The present paper describes the action of various inhibitors of serine-type esterases, as well as the action of iodine on the tomato pectinesterase.

Material and methods. Pectinesterase was prepared from ripe tomatoes (Lycopersicum esculentum, var. Immuna) as described previously[4]: the crude product was gained after extraction, fractional salting out with ammonium sulfate, dialysis and desalting on Sephadex G 25 column. The crude product was further purified on DEAE Sephadex A 50 and by chromatography on Sephadex G 75 column. The fraction with maximal pectinesterase activity was desalted on Sephadex G 25 column, and this product represented the purified pectinesterase. The isolated

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