Table 2. Inhibition of sepiapterin deaminase (%)

Compounds	Concentrations 10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
KF	72.0	18.6				
p-Chloromercuri-benzoate		80.0	11.8	0		
8-Azaguanine		51.3	17.3	0		
Phenyl methyl sulfonylfluoride		32.0	6.4	0		
Amethopterin			76.0	41.5	6.4	0
Aminopterin			83.2	14.3	7.9	0

sepiapterin, 0.4 μmoles; 3.5 units of enzyme, and inhibitor as described. After incubation at 25 °C for 10 min, 0.5 ml of 0.3 N NaOH was added and the decrease in absorbance at 475 nm was determined as previously described[3]. Sodium azide, KCN, monoiodoacetic acid, dinitrophenol, propionic acid, melamine and EDTA were not effective as inhibitors at a final concentration of 10^{-3} M for either type of deaminase preparation. Substances which were effective as inhibitors are listed in table 2. These compounds produced the same degree of inhibition with both type of enzyme preparation, thus indicating that the deaminase activity found in the 2 strains is due to 1 protein. The susceptibility of the enzyme to p-chloromercuribenzoate shows that sepiapterin deaminase differs from rat liver pterin deaminase[5] and Bombyx mori isoxanthopterin deaminase[6]. With 17.5 units of purified sepiapterin deaminase, isoxanthopterin deaminase activity was estimated[6], neither ammonia nor product 7-oxylumazine was detected. These data prove that the 2 enzymes differ from each other. Since bacterial pterin deaminase is inhibited by KF at a concentration of 3×10^{-5} M, it too is distinct from silkworm sepiapterin deaminase.

Lineweaver-Burk plots of normal and inhibited deaminase activity are shown in the figure. It can be seen that amethopterin and 8-azaguanine are competitive inhibitors of the enzyme, while p-chloromercuribenzoate is a non-competitive inhibitor. From the figure, K_i values for the substances were calculated as follows: amethopterin, 1.9×10^{-5} M; 8-azaguanine, 6.7×10^{-4} M; p-chloromercuribenzoate, 2.4×10^{-3} M.

The decreased concentration of sepiapterin deaminase in the integument of the normal type silkworm is noteworthy. In the normal type silkworm, it is probable the sepiapterin is converted to tetrahydrobiopterin via dihydrobiopterin. In the lemon mutant strain, a lack of sepiapterin reductase results instead in the accumulation of sepiapterin. The excess sepiapterin is excreted after deamination by sepiapterin deaminase, an action which produces a compound which is more water-soluble than sepiapterin itself. The high specific activity of the deaminase in malpighian tubules[3] supports this possibility.

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Large scale preparation of calf liver nuclei by continuous flow centrifugation

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Summary. Large scale purification and preparation of calf liver nuclei was accomplished by high speed centrifugation of a fraction enriched in nuclei ('nuclear homogenate') through 1.8 M sucrose by means of a Beckman CF-32 Ti continuous flow rotor. In comparison with methods involving the use of conventional high capacity rotors, larger volumes of homogenate could be processed. This method was used to prepare nuclei from calf liver for the preparation of DNA-dependent RNA polymerases. The use of continuous flow ultracentrifugation avoids time-consuming manipulations, thus allowing handling of large quantities of tissue.

High-density sucrose centrifugation is one of the most effective methods for preparing nuclei of animal cells free of contamination from other subcellular particles[3–5]. This point becomes of crucial importance when investigating enzymes present in multiple forms which are located in different subcellular particles. The low content in normal conditions of such enzymes often requires an enrichment of the selected subcellular particles from large quantities of starting tissue material. Whilst studying DNA-dependent RNA polymerase from different calf organs, we developed a method of preparing nuclei from calf liver in a high yield with a good degree of purity, using a continuous flow ultracentrifugation.

Experimental. Sucrose and $MgCl_2$ were reagent grade. A Beckman CF-32 Ti continuous flow rotor in a model L3–50 Spinco ultracentrifuge was used. The flow through the rotor was maintained by means of a Cole Parmer Masterflex model 7565 high capacity peristaltic pump with variable speed control. The temperature during all experiments was maintained below 4 °C.

Calf liver, obtained from a local slaughterhouse, was cut into pieces and thoroughly washed in a few volumes of

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chilled 0.32 M sucrose, 3 mM $MgCl_2$. The liver fragments, weighing 1400 g, were minced in an ordinary meat mincer and suspended in 5 volumes of the washing solution. The suspension was then homogenized in a Waring Blendor in 800 ml aliquots for 5 min at low speed setting. The homogenate was filtered through 2 layers of cheesecloth and a paddle of glass wool in a Buchner funnel, then centrifuged at 2500 × g for 15 min in the preparative rotor 872 of an I. E. C. B-20A centrifuge.

The crude nuclear pellet was resuspended in a measured volume of 2.4 M sucrose, 1 mM $MgCl_2$ and brought with the same solution to a final volume corresponding to a 5:1 ratio with respect to the pellet volume. The suspension was then stirred in the blendor for 3 min and filtered once again through two layers of cheesecloth and used for the following experiments. This suspension will be referred in the text as 'nuclear homogenate'. DNA-dependent RNA polymerase from calf liver nuclei was prepared and assayed as described earlier[6].

Results and discussion. 2 aliquots of the nuclear homogenate, whose concentration in sucrose was 2.05 M, were diluted with 1 mM $MgCl_2$ to a final sucrose concentration of 1.5 M and 1.7 M respectively. The 2 solutions were used for a set of 2 experiments. While the rotor was running at low speed, the peristaltic pump was used to introduce 125 ml of overlay solution (1.4 M sucrose and 1.6 M sucrose according to the concentration of the nuclear homogenate) followed by 300 ml of the heavier solution, 1.8 M sucrose, 1 mM $MgCl_2$, at the outer edge of the rotor. The rotor was then accelerated to 30,000 rpm corresponding to a maximum force of 90,000 × g and the sample, pumped through the centre lines of the rotor, was introduced at the lower edge of the core at different flow rates. Nuclei were subjected to 75,000–80,000 × g for the time required to reach the top of the core and, at the appropriate flow rate, became trapped in the 1.8 M sucrose layer, eventually pelletting to the rotor wall. The effective removal of nuclei from the samples was checked by collecting the effluent and pelletting residual nuclei over a small 1.8 M sucrose cushion in the JA-21 rotor of a Spinco J21B centrifuge at 50,000 × g for 30 min. At the end of the run, the rotor was brought to a stop, the nuclei were removed from the rotor wall with the aid of one of the Noryl core sector dividing parts and weighed.

As shown in the table, the same recovery of nuclei was obtained in the 2 experiments, indicating that no trapping of the nuclei occurred at the interface between the heavy and light solutions when the sucrose concentration difference between the 2 solutions was enhanced. Such trapping was observed when pelletting at similar conditions in normal centrifuge tubes, and is presumably due to the presence of a sharp boundary between the 2 solutions; it seemed to be prevented, when the continuous flow rotor was used, by the formation of a short, but definite, gradient between the heavy and light solutions when they come in contact within the rotor.

Continuous flow centrifugation of nuclear homogenate

Sample	Sucrose concentration (M)	Maximal flow rate for complete clean out (l/h)	Recovery* (%)
1	1.5	2.7	1.57
2	1.7	2.3	1.66

* Recovery was expressed as wet weight of nuclear pellet to weight of the tissue.

Some difficulties were encountered in pumping the highly viscous 1.7 M sucrose solution into the rotor. Since in both cases nuclei were allowed to sediment through a uniform 1.8 M sucrose layer, the use of the 1.5 M sucrose solution, easier to handle and to pump into the rotor, seemed preferable.

The use of a high density sucrose homogenate restricted the species of sedimenting subcellular particles to the heavier ones and prevented the building up of a layer of particles of intermediate density at the interface between the 1.8 M sucrose layer and the sample. These particles, when processing large volumes, would interfere with the sedimenting nuclei and eventually contaminate them. Phase contrast microscopic examination of the pelletted nuclei revealed intact nuclei with very little contamination. The isolated nuclei were also stained with methyl green-pyronine; no ribosomal contamination could be seen. Nuclei obtained by continuous flow ultracentrifugation were used to prepare DNA-dependent RNA polymerases. The purification procedure consisted of sonication at high ionic strength of nuclei, chromatin removal and ammonium sulphate precipitation. The figure shows the elution profile of enzyme activities from a DEAE-Sephadex A-25 column. The pattern of elution obtained was essentially identical to that observed when the enzyme was prepared from nuclei purified according to Pogo et al.[7].

The high centrifugal force developed in this rotor allows rapid sedimentation of the nuclei even at the high sucrose concentration necessary to prevent contemporary sedimentation of other subcellular particles. The concentration of the layer through which nuclei sediment, can be increased to improve the degree of purity. Of course, at

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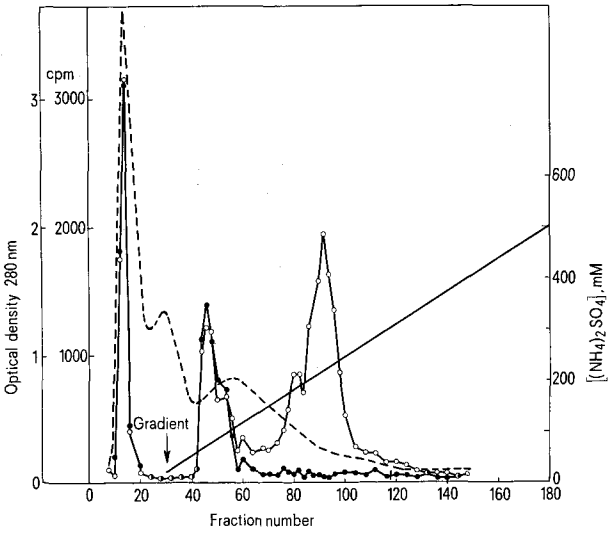

DEAE-Sephadex chromatography of RNA polymerases from calf liver nuclei. 5 ml of a partially purified fraction (204 mg of protein) were loaded on a 1 × 13 cm column equilibrated with 50 mM Tris-HCl, pH 7.9, 25% (v/v) glycerol, 5 mM $MgCl_2$, 0,1 mM EDTA, 5 mM dithiothreitol, 20 mM $(NH_4)_2SO_4$. The column was eluted with 150 ml of a 20–500 mM $(NH_4)_2SO_4$ linear gradient; 1.1 ml fractions were collected and assayed for enzyme activity in the absence (○) or the presence (●) of 1 μg of α-amanitin; (——) ammonium sulphate concentration; (----) absorbance at 280 nm.

concentrations higher than 1.8 M sucrose the yield will decrease progressively; a compromise must be reached according to specific purification needs. If the nuclei content of the homogenate is known, the thickness of the nuclear pellet at the rotor wall can be calculated considering that at 1 mm from the rotor wall a 1 mm band will hold about 41 ml of nuclear pellet. At the conditions described above, processing 10 l of homogenate would result in the formation of a band only about 2 mm thick. Alternatively, a cushion of a denser sucrose solution could be introduced between the 1.8 M sucrose layer and the rotor wall; in this case no pelletting would occur and nuclei, concentrated in a narrow band at the interface with the 1.8 M sucrose layer, could be collected by emptying the rotor with the same procedure employed when a gradient experiment is performed. However, in order to avoid the time-consuming gradient elution step and a second passage of nuclei in concentrated suspension through the rotating seal of the rotor which might lead to damage, pelletting to the rotor wall was preferred.

Amino acid composition and sequence of kassinin, a tachykinin dodecapeptide from the skin of the African frog Kassina senegalensis[1]

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Summary. Methanol extracts of the skin of the African amphibian Kassina senegalensis contain a dodecapeptide, kassinin, belonging to the family of tachykinins or physalaemin-like peptides. Kassinin, like all other natural tachykinins, possesses the characteristic C-terminal tripeptide Gly-Leu-Met-NH_2 and a phenylalanine residue in position 5 from the C-terminus. However, the amino acid sequence in the N-moiety of the molecule differs sharply from that of the other tachykinins.

A new peptide which can be included in the tachykinin family has been traced in the skin of the African frog Kassina senegalensis. The peptide, called kassinin, has been isolated in a pure form and its structure identified as follows: + Asp-Val-Pro-Lys-Ser-Asp-Gln-Phe-Val-Gly-Leu-Met-NH_2.

It may be seen that kassinin possesses the C-terminal tripeptide -Gly-Leu-Met-NH_2 and the Phe residue at position 5 from the C-terminus which are characteristic for all known natural peptides of the tachykinin group (eledoisin, physalaemin, phyllomedusin, uperolein, substance P), and are essential for the physalaemin-like activity.

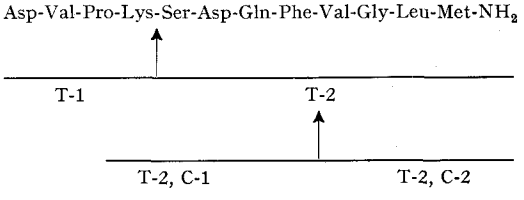
However, kassinin differs from all the other tachykinins in that it possesses 12 amino acid residues, and strikingly differs from the amphibian tachykinins in the amino acid composition of the N-moiety of the molecule. Moreover, like substance P, kassinin shows a free N-terminus, instead of the usual pyroglutamyl residue.

Materials. The fresh skins of 1200 specimens of Kassina senegalensis, collected in Kenia and South Africa during the period 1971–1974 were used in this study. The material weighed 659 g (average 0.55 g per fresh skin). The skins were removed from the frogs immediately after killing and extracted twice with a volume of methanol 5 times the weight of the tissue. The methanol extracts were combined and filtered and then stored in the refrigerator.

Isolation procedure. Samples of pure peptide were obtained by submitting the extracts to the following purification steps: a) washing of the evaporation residue with petroleum ether in order to eliminate fat contaminants; b) 120-tubes countercurrent distribution with the system n-butanol:acetic acid:water (80:12:108); c) gel filtration through Sephadex G 10 columns eluted with 0.01 M acetic acid; d) gel filtration through Sephadex G 25 columns eluted with 0.01 M acetic acid. Further purification could be obtained (when needed) by chromatography on alumina columns and/or preparative electrophoresis on paper.

Purification of kassinin could be followed, step by step, by bioassay (guinea-pig ileum) and by paper electrophoresis. At pH 1.2 the peptide showed an electrical mobility of 0.55 Glu, but no mobility was observed at pH 5.8. Rf values on paper chromatograms were 0.54 in the system n-butanol:ethanol:acetic acid:water (80:16:16:128) and 0.21 in the system n-butanol:ethanol:water (96:16:128). The spot of kassinin could be revealed by ninhydrin and chlorine.

Structure. The occurrence in the kassinin molecule of a lysyl and a phenylalanyl residue (demonstrated by total acid hydrolysis) was the premise for the treatment of the molecule with trypsin and chymotrypsin. By digestion of kassinin with trypsin, 2 fragments were obtained, containing 4 and 8 amino acid residues, respectively. Submitting the octapeptide to chymotrypsin digestion, it split into 2 tetrapeptide fragments, as shown below.



The same 3 tetrapeptide fragments were obtained by reversing the succession of enzymes.

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