assay mixture and resuspended at intervals for 5 min at 0 °C. Free cAMP adsorbed onto the cellulose was removed by centrifugation at 800 × g for 10 min at 0 °C. One ml of the supernatant was mixed with 10 ml of Aquasol-2 (New England Nuclear, Boston, Mass.) and radioactivity was measured in a Packard Tricarb Scintillation Counter with a counting of efficiency of 45% as determined with an external standard.

Effect of washing the sample with different volumes of phosphate buffer on the retention of protein kinase bound cyclic (^{3}H) AMP by the membrane filter

Vol. of phosphate buffer (ml)	Radioactivity (cpm)
5	12774
10	11621
15	11421
20	10235
25	9108
30	8951
35	8887
40	8230

Cyclic ^{3}H AMP (2 pmoles 27.5 Ci/mmole) was incubated with 4 µg of protein kinase; 28 µg of protein kinase inhibitor in 50 mM sodium acetate buffer, pH 4.0 in a total volume of 0.1 ml at 0 °C for 60 min. After incubation, 1 ml of 20 mM sodium phosphate buffer pH 6.0 was added to the reaction mixture and the mixture was allowed to equilibrate for 5 min at 0 °C. The diluted reaction mixture was then filtered over a Millipore filter (25 mM diameter; catalogue No. HAWP. 025). After washing the sample with different volumes of the buffer, the membrane was dissolved in 1.0 ml of Cellosolve mixed with 10 ml of Aquasol 2 and the radioactivity was measured.

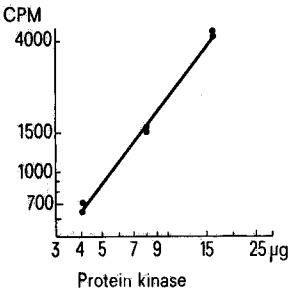

Fig. 2. Effect of protein kinase concentration on the amount of cAMP bound to the enzyme. Varying amounts of protein kinase was incubated in 2 µM labelled cAMP as described in the materials and methods section. Enzyme bound and free cAMP were separated by QAE cellulose suspension.

Results. In the table, the effect of varying the volume of phosphate buffer on the bound cAMP is tabulated. As can be seen, the labelled cAMP decreases progressively with the volume of the buffer employed. This finding indicates that a considerable error can be introduced by relatively small changes in this part of the procedure.

The results of a typical displacement experiment using the QAE cellulose to remove free cAMP are shown in figure 1. As expected, the measured radioactivity decreased as the unlabelled cAMP concentration increased and showed an excellent correlation on a double logarithmic plot. These results are in good agreement with the data of Gilman[2].

In a further experiment, increasing amounts of protein kinase were incubated with 200 pmoles of (^{3}H)-cAMP (50,000 cpm) in otherwise identical mixture described above. After incubation at 0 °C for 60 min, protein kinase bound cAMP was separated by mixing QAE cellulose suspension with the assay mixture and the radioactivity of the supernatant was determined. Figure 2 shows the relationship between the µg of protein kinase added to the reaction mixture and the amount of tritium labelled cAMP bound to the enzyme. It was noted that the amount of cAMP bound to the protein increases with the concentration of protein kinase. At a cAMP concentration of 2 µM, 1.18, 1.98 and 2.81 pmoles of cAMP would bind to 1 µg of protein in the presence of 4, 8 and 16 µg of the enzyme in the assay mixture.

Discussion. The unexpected finding that the volume of washing buffer affects an assay for cAMP led to a quest for a more reproducible and technically simpler method. Separation of protein bound cAMP from the free nucleotide by QAE cellulose suspension is quantitative, and more easily performed than by membrane filtration and, unlike the membrane filtration technique, the QAE cellulose adsorption method does not need any special apparatus. Like QAE cellulose, activated charcoal coated with bovine serum albumin or dextran has also been employed to separate free cAMP from protein kinase bound moiety[3]. However, the extent of adsorption of the free cAMP over cAMP bound to protein kinase by several commercially available activated charcoal varies considerably and the selection of the charcoal has to be carefully made[3]. The variation of recovered counts in replicate samples using the QAE method is minimal. The technique has been successfully utilized for the determinaction of cAMP concentrations in human platelets and lymphocytes.

Serum and liver radioactivity levels in mice after intraperitoneal and subcutaneous injection of [^{14}C]orotic acid[1]

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Summary. [^{14}C]Orotic acid was rapidly distributed in blood after both i.p. and s.c. injection but was not completely absorbed from the peritoneal cavity until 20 min after injection. S.c. injection should be an acceptable alternative to i.p. injection although the incorporation into the liver acid soluble- and RNA-fractions was somewhat delayed after the s.c. injection.

In a previous study, i.p. administration was shown to be as effective as i.v. administration as regards incorporation of [^{14}C]orotic acid into total acid soluble and RNA-fractions in various mouse organs[2]. A problem arising during the analysis of radioactivity levels in metabolites after administration of radionuclides, is the possible interference by unabsorbed radionuclide at the site of injection[2,3]. An i.p. injection may result in a contamination of the liver and other peritoneal organs and i.p. sampled blood is likely to be contaminated to a certain extent by unabsorbed nuclide. In order to ascertain whether these problems could be overcome by using a

Radioactivity recovered from the peritoneal cavity after i.p. injection of [^{14}C]orotic acid and the ratio i.p./s.c. of cpm/g (wet wt) liver in the acid soluble and RNA-fractions

Time after injection (min)	Radioactivity recovered from the peritoneal cavity (% of injected dose)	Ratio $\frac{\text{cpm/g liver after i.p. injection}}{\text{cpm/g liver after s.c. injection}}$	
		Acid soluble fraction	RNA-fraction
2	31.8	6.62	5.44
10	–	1.61	1.46
20	1.6	1.17	1.26
60	–	0.98	1.08

Each value represents the mean of 2 or 3 animals.

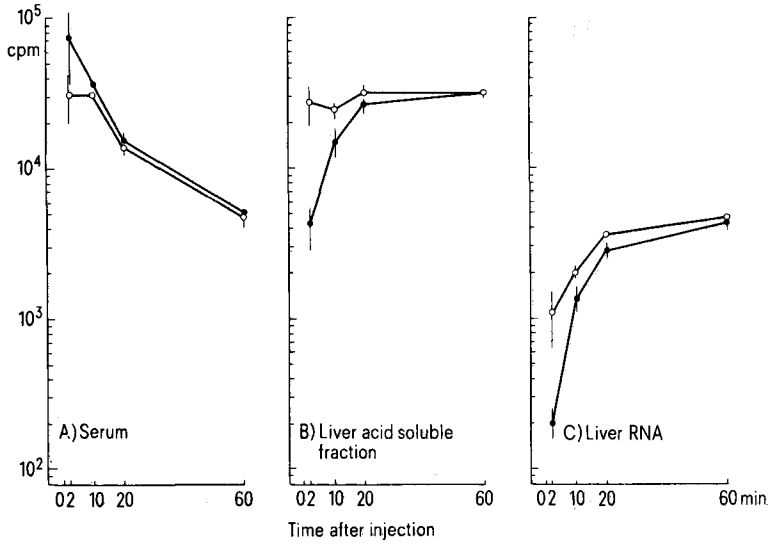
s.c. injection and whether the s.c. administered orotic acid was effectively taken up by the blood and incorporated into the liver, we measured the radioactivity in the liver RNA-fraction and in the acid soluble fractions of the liver and of blood sampled at the brachial vessels 2–60 min after i.p. or s.c. injection of [^{14}C]orotic acid.

Materials and methods. Male NMRI mice (Anticimex, Stockholm, Sweden), weighing 26–28 g, were used for the experiments. They were kept under constant conditions regarding light and temperature and were given standard food and water ad libitum. The radionuclide [6-^{14}C]orotic acid (spec. act 61 mCi/mM, Amersham, England) was administered in 75 µl 0.9% NaCl (1.75 µCi) either i.p. or s.c. in the neck. The animals were killed at 2, 10, 20 or 60 min after injection. 2 min prior to tissue sampling, the animals were anaesthetized in an ether-oxygen atmosphere[4]. The peritoneal cavity was opened and blood was then sampled at the brachial vessels[5]. The exposed liver was rapidly removed, frozen between precooled aluminium blocks and immersed in liquid nitrogen. The blood samples were allowed to coagulate and the serum was isolated and acidified with PCA. The liver tissue was homogenized and fractionated into an acid soluble and a RNA-fraction according to Munro and Fleck[6]. In an experiment designed to measure the amount of radionuclide remaining in the peritoneal cavity after i.p. injection, the animals were killed by decapitation 2 or 20 min after injection. Following exsanguination, the peritoneal cavity was opened, washed 3 times with 1 ml distilled water and the radioactivity in the pooled water was measured. All radioactivity measurements were made in a Packard TRI Carb liquid scintillation spectrometer.

Results and discussion. The [^{14}C]orotic acid was rapidly distributed in the blood of both the i.p. and the s.c. injected groups of animals and the levels of radioactivity in serum sampled at the brachial vessels were similar in both groups (figure, A). The tendency towards a higher level of radioactivity in serum after s.c. injection could possibly be explained by the short distance between the site of injection and the site of blood sampling[7]. Alternatively since the orotic acid would reach the liver cells more directly after i.p. administration, it may be more rapidly extracted from the blood due to the pronounced ability of the liver to accumulate orotic acid[8]. The liver acid soluble radioactivity at 2 and 10 min after i.p. injection was well above that after s.c. injection (figure, B). This observation could be explained by a contamination of the liver and/or a more effective incorporation of the i.p. administered radionuclide into the liver cells. An enhanced incorporation into the liver cells seems to be dominating, as indicated by the higher level of radioactivity in the liver RNA-fraction at 2 and 10 min after injection (figure, C). Since penetration into tissues by diffusion is considered to be rather slow[9], it is probable that the i.p. administered radionuclide, by entering the peritoneal capillaries, was more directly and rapidly distributed among the liver cells by the portal vessels than the s.c. administered radionuclide.

2 min after i.p. injection, about 30% of the administered radioactivity could be retrieved by washing the peritoneal cavity with distilled water (table). Less than 2%

1 This work was supported by grants from the Carl Trygger Foundation and the Foundation of Director Albert Påhlsson.
2 L. Lewan, I. Petersen and T. Yngner, Hoppe Seylers Z. physiol. Chem. *356*, 425 (1975).
3 T. Yngner, L. Lewan and I. Petersen, Experientia *31*, 387 (1975).
4 N. L. R. Bucher and M. N. Swaffield, Biochim. biophys. Acta *129*, 445 (1966).
5 L. Young and T. R. Chambers, Lab. Anim. Sci. *23*, 428 (1973).
6 H. N. Munro and A. Fleck, Analyst *91*, 428 (1966).
7 C. G. Potter, Experientia *30*, 25 (1974).
8 R. B. Hurlbert and V. R. Potter, J. biol. Chem. *209*, 1 (1954).
9 S. R. Pelc and T. C. Appleton, Nature *205*, 1287 (1965).



Radioactivity per ml serum (A) and per g (wet wt) liver in the acid soluble (B) and RNA-fractions (C) after i.p. (○) and s.c. (●) injection of [^{14}C] orotic acid. Each point represents the mean of 2 animals. Highest and lowest values are indicated or within the symbols. Semilogarithmic scale.

of the administered radioactivity was retrieved 20 min after injection. It seems likely that the high level of liver acid soluble radioactivity 2 min after i.p. injection partly depends on a contamination by unabsorbed radionuclide. This is supported by the 20% difference between the ratios of i.p. to s.c. administered radioactivity in the liver acid soluble and RNA-fractions (table). Although the incorporation of labeled orotic acid into the RNA-fraction after s.c. injection lagged behind that after i.p. injection only a minor difference was found between the levels reached at 60 min after injection. This should depend on the high capacity of the mouse liver to accumulate orotic acid[2]. Our result show that in order to avoid contamination of the liver acid soluble fraction and of i.p. sampled blood by unabsorbed radionuclide after short pulse-periods, i.p. injection should be avoided. It is evident that s.c. injection in the neck is preferable as it in addition to an effective incorporation of radioactivity into the liver cells also allows i.p. sampling of blood, a fact that is of great importance when hypoxia must be minimized during liver tissue sampling[10].

10 R. P. Faupel, H. J. Seitz and W. Tarnowski, Archs Biochem. Biophys. *184*, 509 (1972).

Keto acids and free amino acids during leaf growth in Bauhinia purpurea L.

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Summary. The biosynthesis of keto acids and free amino acids was studied during the growth of Bauhinia purpurea leaves. α-KGA, OAA, pyruvic acid and PEP are the important keto acids observed at various stages. The first 2 metabolites show a progressive increase and PEP → OAA pathway is very active during the process of growth.

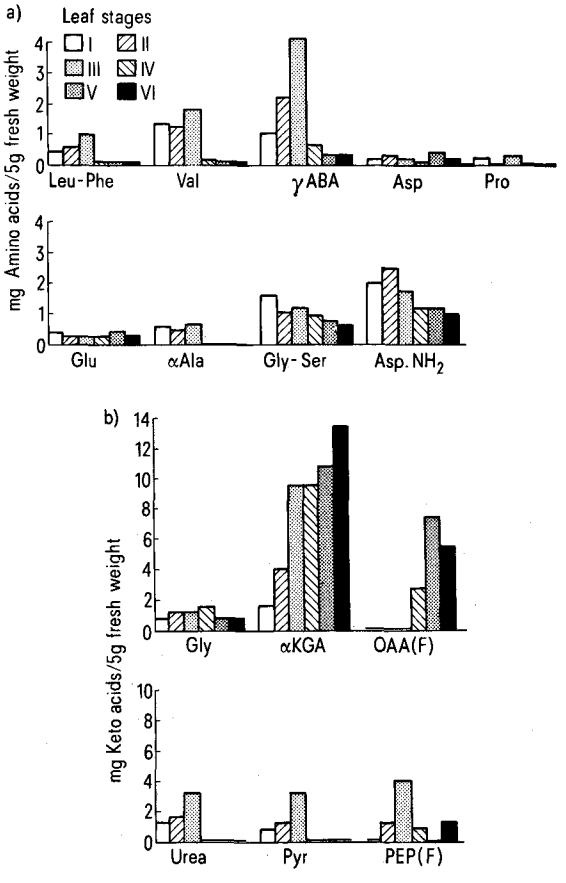
The involvement of amino acids and keto acids in interrelated metabolic pathways has been indicated since long[3]. Changes in the amino acids and proteins with the growth and development of the leaves have been worked out in detail, but little is known about the changes in keto acids in maturing leaves. The present study is aimed at studying changes in keto acids concentrations and correlating with that of amino acids during leaf maturation.

Material and methods. Bauhinia purpurea plant growing in the University campus has been selected for this study. Leaves of different stages (2-, 4-, 6-, 9-, 12- and 15-day-old) were plucked from the tree, brought to the laboratory and analyzed.

The method described by Steward, Wetmore, Thompson and Nitsch[4] has been followed for amino acid extraction. 2-dimensional paper chromatography has been used for their separation. The detailed procedure is same as that described by Pal and Laloraya[5]. Milligrams of different amino acids are calculated in terms of glycine, using a Klett photoelectric colorimeter.

Keto acids have been extracted as 2,4-dinitrophenyl hydrazones (2,4-DNP's) as described by Kaushik[6] and Mukherjee[7,8]. For the separation of keto acids also, 2-dimensional paper chromatography has been employed. Milligrams of different keto acids are calculated in terms of 2,4-DNP of α-ketoglutarate (α-KGA) using a Klett photoelectric colorimeter fitted with blue filter.

Results. The figure shows that with the development of leaves the level of asparagine shows a gradual decline after the stage II, while glutamine is present only in trace



B. purpurea. Showing levels of amino acids and keto acids in different stages of leaves. *a* Amino acids: Leu & Phe, leucine and phenylalanine; Val, valine; γ-ABA, γ-amino butyric acid; Pro, proline; Ala, α-alanine; Glu, glutamic acid; Asp, aspartic acid; Gly & Ser, glycine and serine; Asp·NH_2, asparagine. *b* Keto acids: Gly, glyoxylic acid; OAA (F), oxaloacetic acid (fast-moving isomer); α-KGA, α-ketoglutaric acid, urea; Pyr, pyruvic acid; PEP (F), phosphoenolypyruvic acid (fast-moving isomer).

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2 Department of Life Sciences, Indore University, Indore, India.
3 R. M. Krupka and G. H. N. Towers, Bot. Rev. *25*, 108 (1959).
4 F. C. Steward, R. H. Wetmore, J. F. Thompson and J. P. Nitsch, Am. J. Bot. *41*, 123 (1954).
5 R. N. Pal and M. M. Laloraya, Physiol. Pl. *20*, 789 (1967).
6 D. D. Kaushik, Ph. D. Thesis, University of Allahabad, India (1966).
7 D. Mukherjee, Ph. D. Thesis, University of Allahabad, India (1972).
8 D. Mukherjee, J. Indian Bot. Soc. *53*, 115 (1974).