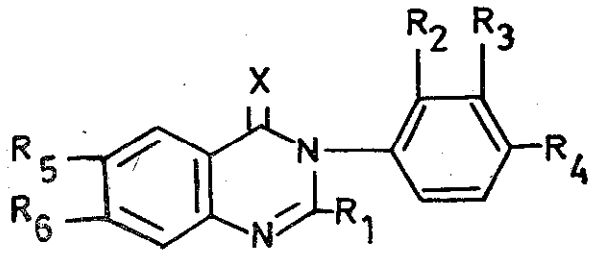
TABLE 1—COMPOUNDS SCREENED FOR THEIR EFFECT ON PYRUVIC ACID OXIDATION IN RAT BRAIN HOMOGENATES

(Each experiment was done in duplicate. Results represent mean values of per cent inhibition calculated from 2 separate experiments. Inhibition was determined by the decrease in O_2 uptake/125 mg wet weight of tissue/hr. All compounds were used at a final concentration of 2m*M*)



2-ALKYL-3-ARYL-6-/7-FLUORO-4(3H)-QUINAZOLONES AND THIOQUINAZOLONES

Compound No.	R_1	R_2	R_3	R_4	R_5	R_6	X	Inhibition %
1	CH_3	H	H	H	F	H	O	17.74
2	CH_3	H	F	H	F	H	O	11.64
3	CH_3	H	F	H	F	H	S	34.51
4	CH_3	H	H	F	F	H	O	56.59
5	CH_3	H	H	CCH_3	F	H	O	17.84
6	CH_3	H	H	OC_2H_5	F	H	O	41.99
7	CH_3	H	H	OC_2H_5	F	H	S	36.78
8	C_2H_5	H	H	H	F	H	O	66.25
9	C_2H_5	H	F	H	F	H	O	15.61
10	C_2H_5	H	H	F	F	H	O	5.85
11	C_2H_5	H	H	Cl	F	H	O	17.68
12	C_2H_5	H	CF_3	H	F	H	O	29.93
13	C_2H_5	H	CH_3	H	F	H	O	67.22
14	C_2H_5	H	CH_3	H	F	H	S	50.44
15	CH_3	H	H	H	H	F	O	80.30
16	CH_3	H	H	H	H	F	S	29.76
17	CH_3	H	H	Br	H	F	O	16.74
18	CH_3	H	CF_3	H	H	F	O	55.74
19	CH_3	H	CF_3	H	H	F	S	5.18
20	CF_3	H	H	H	F	H	O	4.66

(Nos 15, 16 and 18), showed promising inhibitory effects.

Compounds having a fluorine substituent either in 6- or 7-position with a 2-methyl substituent (Nos 3, 4, 6, 7, 15, 16, and 18) and a 2-ethyl substituent (Nos 8, 12, 13 and 14) showed significant activity, while a compound having a 2-trifluoromethyl substituent (No. 20) showed very poor inhibitory effect. This activity is very low as compared to its 2-methyl analog (No. 1) and 2-ethyl analog (No. 8). Thus, the replacement of 2-CH_3 or 2-C_2H_5 group by a 2-CF_3 group decreases the activity markedly.

In the quinazolone series, a marked decrease in CNS activity, has been observed when a C=O group was replaced by a C=S group in 4-position of the quinazolone ring system[4]. In this series, the replacement of a C=O group by a C=S group in 4-position of the quinazolone ring system also resulted in marked decrease in inhibition (compare the pair of compounds: 6, 7; 13, 14; 15, 16; 18, 19—Table 1) except compound No. 3 as compared to No. 2.

Quinazolones, having an unsubstituted phenyl ring in 3-position, displayed significant activity (compounds No. 8 and No. 15) except compound No. 1. Similarly, a few of the 3-substituted phenyl derivatives (compounds No. 4, No. 13 and No. 18) were found to be highly active. From these data, it is difficult to make a definite generalization about the role of substituents in the 3-phenyl ring.

The general observations are that fluorine substitution, either in 6- or 7- position, results in enhancing the inhibitory effect up to some extent.

The authors thank Prof. R. C. Mehrotra, Head of the Department, for providing facilities and Prof. S. S. Parmar, Department of Pharmacology, K. G. Medical College, Lucknow, for help in evaluating the biological activity of the compounds.

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Isolation & Determination of Adenosine 3′, 5′-Cyclic Monophosphate from Plant Tissues

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Manuscript received 28 *July* 1973; *revised manuscript received* 12 *September* 1973

A procedure has been described to determine picomole levels of adenosine3′,5′-cyclic monophosphate (CAMP) in plant materials. The purification of CAMP from other interfering nucleotide and phenolic compounds is made using paper and column chromatography. The resulting CAMP is enzymatically transformed to ATP and estimated by its luminescent reaction with luciferase. The advantages of this method have been discussed in the light of purification and assay of CAMP. The content of CAMP in lettuce, barley seeds, cabbage leaves, and cultivated mushrooms has been estimated by this procedure.

THE discovery of adenosine 3′,5′-cyclic monophosphate (CAMP) in liver tissues[1] has initiated a number of investigations on its distribution in mammalian tissues[2-4], bacteria[5-7], slime molds[8] and plant tissues[9-12]. Although the biological role of CAMP is well documented in animals, bacteria and slime molds, no intensive studies have been reported in the case of higher plants, probably for the following analytical difficulties : (a) concentration of CAMP is extremely low as compared to that of the other nucleotides present in higher plants; (b) separation of such low amounts of CAMP from other potentially interfering nucleotides; and (c) interference of phenolic compounds present in the extract with the enzymatic assay of CAMP.

Taking all these analytical difficulties into account, a practical and sensitive method for the

estimation of CAMP has been developed, especially for plant tissues.

The method, as described in this paper, involves fixation, extraction and separation of CAMP from other interfering nucleotides by paper chromatography, elimination of phenolic compounds by the use of column chromatography (Dowex–50–H^+or anhydrous aluminium oxide) and finally enzymatic estimation. The validity of the method is checked on lettuce, barley seeds, cabbage leaves, cultivated mushrooms and rat lung.

Plant tissues (0.2 to 1.0 g) were frozen in liquid nitrogen and ground with an ultra-turrax at —20°C with 10 ml of ethyl ether containing 0.98 g of trichloroacetic acid (TCA) directly in a stainless steel centrifuge tube held in an alcohol bath at —20°C. The temperature of the fixed sample was slowly raised to 0°C and the homogenate was centrifuged. The supernatant was collected in a separating funnel kept at 0°C. The residue was extracted with 10 ml of 0.1 *N* TCA and centrifuged; the supernatant was added to the first ether extract. TCA was eliminated by shaking with cold ether. ^{3}H-CAMP, (0.02 μCi, sp. activity 18 Ci/mmole; obtained from Dr Fromageot, C.E.A., Paris) was added to TCA free extract which was then concentrated to approximately 1 ml by evaporation under reduced pressure at 35°C and was used directly for paper chromatography.

Descending paper chromatography on Whatman 3MM paper was used. The 1 ml concentrate was applied as a narrow band along the starting line. The solvent, isopropanol-conc. NH_4OH-H_2O (7 : 1.5 : 1.5 vol vol), was allowed to reach the lower end of the paper within 20 hr at 24°C in a constant temperature room. The chromatograph was air dried and the location of CAMP was detected by the presence of ^{3}H-CAMP. The strip corresponding to CAMP was eluted with 50% ethanol and concentrated under reduced pressure at 35°C to 0.5 ml. The concentrated solution was yellowish in colour.

The concentrated solution containing CAMP and the remaining non-cyclic adenine nucleotide and phenolic compounds was purified by one of the two following procedures.

Ion-exchange chromatography — The concentrated solution (about 0.5 ml) was chromatographed on Dowex-H^+ (50W 200-400 mesh) column[13] (6×0.6 cm) and was eluted with distilled water. The first 3 ml fraction contained almost all inhibiting substances which inhibit the enzymatic assay of CAMP. The next 4 ml fraction contained the CAMP and the column retained traces of 5'-AMP if present in the extract.

Adsorption chromatography — The concentrated solution (about 0.5 ml) was passed through a column of anhydrous aluminium oxide prepared with disposable Pasteur pipets[14]. The column was eluted with 10 m*M* tris-HCl buffer (*p*H 7.4). The first 2 ml fraction contained all the CAMP. The compounds that interfere with the subsequent enzymatic assay were retained by the column.

The eluates obtained either from ion-exchange or aluminium oxide chromatography were concentrated under reduced pressure to dryness and taken in 0.2 to 0.5 ml of distilled water. The enzymatic estimation of CAMP was done on this final purified colourless extract. An aliquot of this extract was counted in a Beckman LS 100 Liquid Scintillation system and the coefficient of loss was calculated.

Enzymatic estimation of CAMP — CAMP present in the extract was hydrolysed to 5'-AMP by incubation with 3'-5'-cyclic nucleotide phosphodiesterase from beef heart[15,16]. The resulting 5'-AMP was enzymatically converted into ATP on incubating with adenylate kinase, pyruvate kinase and phosphoenolpyruvate[17] (enzymes were bought from Sigma Chemical Co.). The level of ATP was determined using a crude extract of firefly tails[17]. The assay can detect amounts as low as 0.5 pmole of ATP[10]. Each sample was assayed in duplicate with and without phosphodiesterase plus an added standard of CAMP. To a 30 μl sample was added 20 μl of reaction mixture containing tris-buffer 0.35 mole (*p*H 7.4), $MgSO_4$, 1.1 mole; K_2SO_4, 1.9 mole; phosphoenolpyruvate, 1.7 mole; EDTA 0.02 mole; adenylate kinase; 6.25 μg; pyruvate kinase, 6 μg, and phosphodiesterase, 20 mU when present. The reaction mixture was incubated first at 37°C for 30 min then at 25°C for 1 hr. It was found that the conversion of CAMP to AMP takes place at 37°C and the succeeding conversion was complete if the incubation was carried out at 25°C for 1 hr. When the incubation was done at 37°C for 1½ hr continuously a slight hydrolysis of ATP was noticed. At the end of the incubation, ATP levels were determined. The final result was corrected for the loss of CAMP during the extraction process.

Cyclization of adenylate nucleotides — The possibility of CAMP formation from cyclization of ATP, ADP and AMP during extraction and separation was verified by the addition of ATP, ADP and AMP labelled with ^{32}P in the α-position (labelled nucleotides were obtained from C. E. A., France). For that purpose, 1 g of lettuce seeds was imbibed in distilled water for 2 hr at 20°C and fixed with liquid nitrogen as described above. After fixation, extraction and separation, ATPα^{32}P (10^7 cpm), ADP α^{32}P (10^6 cpm) and AMPα^{32}P (10^6 cpm) were added. Immediately after the separation of CAMP on paper chromatography, the spot corresponding to CAMP was eluted and counted. No radioactivity was detectable in this spot.

The analytical procedure described was utilized to measure the levels of CAMP in surface sterilized imbibed lettuce and barley seeds, cabbage leaves, cultivated mushrooms and rat lung. The results are as follows :

Tissue	CAMP (pmole)
Lettuce seeds	310[a]
Barley seeds	420[a]
Cabbage leaves	2000[b]
Cultivated mushrooms	250[b]
Rat lung	7700[b]
Rat lung[18]	7200[b]

[a]per gram dry weight [b]per gram wet weight

Since the levels of CAMP in plant tissues are low as compared to those of animal tissues[18-21] and bacteria[6] the amount of plant tissue needed for analysis is large. However, we could easily proceed with a sample of 0.25 g because of the simplified procedure that we have developed. The loss during the analysis was estimated from isotopic dilution

using ^{3}H-CAMP. The recovery varied from 30 to 40%.

The paper chromatography step is labourious, nevertheless, it is highly necessary in the case of plant tissues to remove salts and the major part of non-cyclic adenine nucleotides and phenolic compounds. The solvent system used in the paper chromatography has two advantages : (1) Because of its volatile nature, the CAMP is obtained free of salts and other chemicals. (ii) The CAMP is separated from all other possibly interfering adenine nucleotides and particularly adenosine diphosphate ribose (ADPR) which can liberate 5'-AMP by the action of cyclic nucleotide phosphodiesterase[19,20]. The R_f values of ATP, ADP, AMP, CAMP and ADPR in this solvent system are 0.05, 0.11, 0.25, 0.55 and 0.15 respectively. Since enzymatic estimation of CAMP involves a phophodiesterase, it is preferable to use this particular solvent for the separation.

The extract eluted from the paper chromatography was always yellowish in colour because of the presence of phenolic compounds. The final purification step on the Dowex-50W column or Al_2O_3 column removes the inhibitory substances. When the enzymatic assay was performed in the presence of a known amount of CAMP, no inhibitory effect was observed. Also, when phosphodiesterase was not added to the incubation mixture practically no nucleotide could be detected. This clearly shows that the purification achieved with this method is perfectly adequate for the enzymatic analysis of CAMP.

CAMP values for rat lung as determined by this procedure in comparison with those obtained by the phosphorylase activation assay[18] are given above. The two values are in good agreement.

Because our solvent for paper chromatography contains concentrated ammonia, the possibility of CAMP formation by cyclization of ATP, ADP or 5'-AMP had to be considered. It has been noted that there was no formation of CAMP from none of the adenylate nucleotide.

Thus, this analytical procedure can be safely employed for the determination of CAMP in all tissues.

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Evidence of Endoreduplication in Callus Cultures of *Crepis suffreniana* Steud.

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Manuscript received 27 *August* 1973; *revised manuscript received* 1 *November* 1973

Cytology of callus cultures initiated from shoot and leaf tissues of *C. suffreniana* on agar B5 medium with $2.25\times10^{-6}M$ 2, 4-D, was studied. Initially, only diploid cells were present, and later tetraploid, octaploid and aneuploid cells were also present. A mutant culture was isolated and was found to be highly polyploid with chromosome numbers ranging from 22-52. These and other findings suggest that the occurrence of endoreduplication *in vivo* is not a prerequisite for it to occur *in vitro*.

Partanen[1,2] has postulated that the nuclear behaviour of plant tissues cultured *in vitro* is in large part a reflection of their *in vivo* potentialities. Thus a plant tissue culture can be expected to become polyploid provided that endoreduplication occurs *in vivo*, and that the conditions to elicit such a response are present *in vitro*. Conversely, if the differentiation of the tissues *in vivo* occurs without endoreduplication, the tissue cultures may be expected to remain diploid. A survey of the literature shows that in most of the tissue cultures studied cytogenetically, such a relationship does exist[1-8]. However, there are a few notable exceptions. Endoreduplication is known to occur in *Medicago sativa* L.[4], *Glycine max* L.[4], *Haplopappus gracillis* Nutt. (Gray)[3], and *Vicia hajastana* Grossh.[3] tissues *in vivo*. The tissue cultures of these species have been demonstrated to possess only diploid[5,6] or a small frequency of polyploid[3,6,7] cells. On the other hand, *Crepis capillaris* (L.) Wallr. tissues do not undergo endoreduplication *in vivo*. A high incidence of polyploidy, however, has been reported in the tissue cultures of this species[8,9].

In the present study, the chromosome numbers of callus cultures of *Crepis suffreniana* Steud., a species which most likely differentiates without endoreduplication, were recorded over a period of 18 months to find out if these cultures would become polyploid.

Callus cultures were initiated from shoot and leaf tissues of *C. suffreniana* on agar B5 medium[10] containing $2.25\times10^{-6}M$ 2,4-D. The calli were subcultured every 5-6 weeks and kept in a culture room with a constant temperature (27-28°C) and light (2000 lux).

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