Phytochemistry, Vol. 25, No. 11, pp. 2639–2646, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

RADIOIMMUNOASSAY DETERMINATION OF SIX OPIUM ALKALOIDS AND ITS APPLICATION TO PLANT SCREENING

UTE WIECZOREK, NAOTAKA NAGAKURA,* CHRISTIAN SUND, STEFAN JENDRZEJEWSKI and MEINHART H. ZENK

Lehrstuhl für Pharmazeutische Biologie, Universität München, Karlstr. 29, D-8000 München 2, West Germany; *Kobe Women's College of Pharmacy, Kobe 658, Japan

(*Received* 3 *January* 1986)

Key Word Index—*Papaver somniferum*; Papaveraceae; radioimmunoassay; (*S*)-, (*R*)-reticuline; salutaridine; thebaine; codeine; morphine; screening for poppy alkaloids.

Abstract—Radioimmunoassay procedures were developed for the independent and specific determination of sub-nmole quantities of (*S*)- and (*R*)-reticuline, salutaridine, thebaine, codeine and morphine. Assay parameters for all six poppy alkaloids are given and the synthesis of haptens and tracers in the Ci/mmol range is described. These assays were used to screen herbarium material of 100 *Papaver* species and to analyse *P. somniferum* plant populations for alkaloid breeding purposes. The time course of alkaloid appearance during the germination of poppy seeds was also studied.

INTRODUCTION

Since 1976, when we introduced radioimmunoassay (RIA) into phytochemical research [1], numerous RIA tests have been set up to quantify secondary plant products [2]. RIAs are extremely sensitive and efficient analytical tools to determine low M_r compounds quantitatively without the need for purification. A particularly significant application is the screening of all kinds of plant material for pharmacologically active compounds. Two of the pharmaceutically most important plant products are the isoquinoline alkaloids codeine and morphine [3]. Immunoassay kits, commercially available, have been used for the quantification of these compounds in humans [4] and plants (e.g. [5–7]). So far only one RIA has been designed for the determination of morphine in *Papaver somniferum* capsules [8], which, however, showed considerable cross-reactivity towards codeine and thebaine. Our aim was to develop specific RIAs for the *Papaver* alkaloids (*S*)- and (*R*)-reticuline, salutaridine, thebaine, codeine and morphine which should be useful for further biosynthetic and biological investigations such as monitoring biosynthetic events of morphine-type alkaloid formation during *P. somniferum* seedling development, localization of alkaloids by means of cytological techniques in cell types and organelles, screening of cell cultures, selection of overproducing field-grown plants and analysis of herbarium material. Furthermore, this technique would serve as a preparation for the detection of specific enzymes involved in the morphine biosynthetic pathway.

RESULTS AND DISCUSSION

The preparation of haptens, tracers and assay parameters was an adaptation of the protocol developed by Stöckigt and co-workers [9] for the synthesis of scopolamine-containing haptens. The *N*-2-carboxyethyl derivatives of (*S*)- and (*R*)-norreticuline, northebaine, norcodeine and normorphine were synthesized. In the case of salutaridine, the salutaridine-7-(*O*-carboxymethyl)oxime was prepared. Each of these derivatives was coupled to bovine serum albumin in aqueous solution using a water-soluble carbodiimide reagent, to yield immunogens against which antibodies were raised in rabbits. Antibodies from several animals were screened for high specificity using tritiated parent compounds with high specific activities. Only in the case of salutaridine was tritiated salutaridinol I [10] used instead.

The details of the RIA performance in our laboratory have been described previously [1, 9]. In all cases, the antigen–antibody reaction was not pH-dependent in the range 5.5–8.0. Optimal results were obtained by incubating with phosphate-buffered physiological saline (pH 7.4). A selective separation of antibody-bound antigen from free antigen was achieved by precipitating the immunoglobulin fraction with 50% saturated $(NH_4)_2SO_4$ at room temperature. Under these conditions non-specific binding was found to be between 1.3 and 2.0%. The general assay parameters are given in Table 1.

The affinity constants for all six tests were obviously in the same order of magnitude, whereas the antibody concentrations of the six sera (and therefore also the antibody titre) varied markedly. The amount of labelled antigen per test differed by about one order of magnitude, owing to different specific activities. The tracer concentration was reduced to a minimum because high concentrations of labelled antigen lead to loss of sensitivity. The total radioactivity per assay was, however, in each case between 5000 and 10000 cpm.

All six RIAs showed a high degree of sensitivity. They allowed the detection of alkaloids in the fmol and nmol range which was a prerequisite for the planned investigations (Fig. 1). The following example illustrates the sensitivity of the test using the thebaine specific antibody. In 10 ml of extract derived from 1 mg (dry wt) of a plant sample the thebaine concentration could be determined with an accuracy down to a level of 10^{-3}%.

Another prerequisite for successful application of RIA in phytochemical research is specificity. Only if the

Table 1. List of general assay parameters for the RIA of six opium alkaloids

Parameter	(S)-Reticuline	(R)-Reticuline	Salutaridine	Thebaine	Codeine	Morphine
Maximal affinity constant of antiserum (l./mol)	2.5×10^9	0.4×10^9	0.9×10^9	1.8×10^9	8.8×10^9	2.3×10^9
Concentration of antibody binding sites of serum (nmol/l.)	6	60	162	744	380	6
Serum dilution in test	1:72	1:180	1:540	1:2700	1:10800	1:99
Amount of labelled antigen per assay (pmol)	1.4	3.2	3.9	3.1	0.8	0.4
Specific radioactivity of tracer (Ci/mmol)	7.5	3.0	3.1	3.1	20	24
Non-specific binding (%)	1.5	2.0	1.5	1.8	1.3	1.5
Detection limit*	0.020 ng = 55 fmol	0.075 ng = 205 fmol	0.230 ng = 633 fmol	0.055 ng = 158 fmol	0.025 ng = 75 fmol	0.024 ng = 75 fmol
Measuring range (ng)	0.05–5	0.1–10	0.5–50	0.1–10	0.05–5	0.05–5
$B/B_0 = 50\%$ (ng)	0.51	1.19	2.12	0.92	0.27	0.20
Slope of logit/log plot	−2.09	−2.28	−2.54	−2.36	−2.44	−2.89

*As free base.

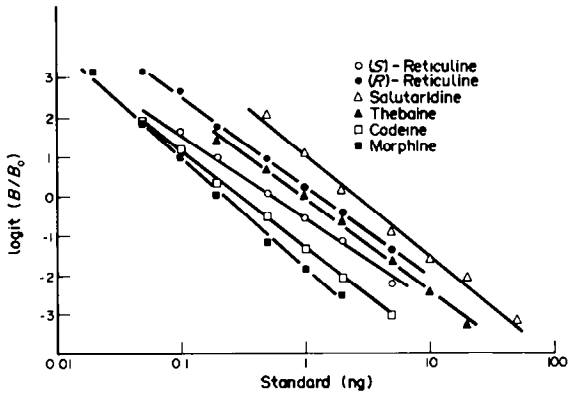

Fig. 1. Comparison of standard curves of RIAs directed against the six opium alkaloids. Assay conditions as given in the Experimental.

antibodies specifically bind the target substance in the presence of structurally similar compounds can the method be applied to crude extracts; this in turn increases the capacity of the test.

The assay specificity was examined with most of the known natural benzylisoquinoline- and morphine-type alkaloids. The results are listed in Table 2. The antibodies against one enantiomer of reticuline completely discriminated between both stereoisomeric forms. Only if the wrong enantiomer was present in a 1000-fold higher concentration could the determination of the target reticuline enantiomer be adversely influenced. A cross-reaction was observed only with the *nor*-compounds. This can easily be explained since the reticuline nitrogen atom is attached to the protein via a carboxyethyl linkage which leads to poor recognition at this site of the molecule. The *S*-specific reticuline antiserum cross-reacts slightly with only two compounds: (*R*,*S*)-4′-*O*-methylnorlaudanosoline (0.8%) and (*S*)-protosinomenine (0.1%). Both alkaloids possess the same C-ring substitution as reticuline, which suggests that the antibodies do not appreciably recognize the A-ring. Conversely, the (*R*)-reticuline-directed antibodies seemed to react more sensitively to differences in the A-ring. No cross-reactivities were observed between the benzylisoquinoline- and the morphinane-type groups.

Within the morphinane group, the salutaridine-directed antibodies were found to cross-react strongly with salutaridinol I (424%), salutaridinol II (171%) and weakly with thebaine (0.3%). The interference with the salutaridinols indicates that the salutaridine skeleton was not clearly recognized at the region around C-7, where it is fixed to the immunogen. Moreover, salutaridine has been replaced by salutaridinol I as tracer, which probably competes better than salutaridine itself. In the thebaine assay, a slight cross-reaction (0.3%) took place only with the closely related oripavine (hydroxyl instead of methoxyl at C-3). The codeine test exhibited measurable cross-reactivities only with the very similar codeinone (0.4%) and with morphine (0.4%). In other words, a concentration of morphine 200-fold higher than that of codeine would interfere with the determination of the latter.

The examination of the morphine antiserum revealed a relatively large cross-reactivity with oripavine (6.2%), probably due to the identical A-ring structure in both molecules. Codeine, however, had no influence on the morphine assay.

Table 2. Cross-reactivities on a molar basis (in %) of six antisera directed against six opium alkaloids

Compound	Antibodies directed against: (*S*)-Reticuline	(*R*)-Reticuline	Salutaridine	Thebaine	Codeine	Morphine
(*S*)-Norlaudanosoline	0	0	0	0	0	0
(*R*)-Norlaudanosoline	0	0	0	0	0	0
(*R,S*)-6-*O*-Methylnorlaudanosoline	0	0	0	0	0	0
(*R,S*)-7-*O*-Methylnorlaudanosoline	0	0	0	0	0	0
(*R,S*)-5'-*O*-Methylnorlaudanosoline	0	0	0	0	0	0
(*R,S*)-4'-*O*-Methylnorlaudanosoline	0.8	0	0	0	0	0
(*R,S*)-Laudanosoline	0	0	0	0	0	0
(*S*)-Norreticuline	5.0	0	0	0	0	0
(*R*)-Norreticuline	0	35	0	0	0	0
(*S*)-Reticuline	100	0	0	0	0	0
(*R*)-Reticuline	0	100	0	0	0	0
(*R,S*)-Nororientaline	0	2.4	0	0	0	0
(*R,S*)-Orientaline	0	7.4	0	0	0	0
(*S*)-Norprotosinomenine	0	0	0	0	0	0
(*R*)-Norprotosinomenine	0	0.6	0	0	0	0
(*S*)-Protosinomenine	0.1	0	0	0	0	0
(*R*)-Protosinomenine	0	0	0	0	0	0
(*R,S*)-Norisoorientaline	0	0	0	0	0	0
(*R,S*)-Isoorientaline	0	0	0	0	0	0
(*R,S*)-Laudanidine	0	0.7	0	0	0	0
(*R,S*)-Tetrahydropapaverine	0	0	0	0	0	0
(*R,S*)-Laudanosine	0	0	0	0	0	0
(*S*)-Coclaurine	0	0	0	0	0	0
(*R*)-Coclaurine	0	4.3	0	0	0	0
Salutaridine	0	0	100	0	0	0
Salutaridinol I	0	0	424	0	0	0
Salutaridinol II	0	0	171	0.2	0	0
Northebaine	0	0	0.1	50	0	0
Thebaine	0	0	0.3	100	0	0
Oripavine	0	0	0	0.3	0	6.2
Codeinone	0	0	0.1	0.2	0.4	0
Norcodeine	n.d.	n.d.	n.d.	n.d.	40	0
Codeine	0	0	0	0	100	0
Normorphine	n.d.	n.d.	n.d.	n.d.	0.2	55
Morphine	0	0	0	0	0.4	100

n.d., Not determined.

In none of the assays was any cross-reactivity observed with dopamine and its derivatives, with protoberberines or with the poppy alkaloids narcotine and narceine.

In order to prove the accuracy of RIA determinations and to observe the magnitude of cross-reactivity within biological samples, the following experiment was carried out. A 90-day-old poppy plant was extracted and the total alkaloid fraction was isolated by absorption onto XAD with subsequent elution. During the isolation care was taken that no immunoreactive material was lost. An aliquot (2.5%) of this crude alkaloid mixture was subjected to HPLC separation as shown in Fig. 2. Each fraction was assayed with the six alkaloid RIAs. The result again proved the high specificity of the RIAs. Morphine did not interfere with the codeine determinations (even if it was present in a 20-fold higher concentration) and there was perfect discrimination between morphine and thebaine. Only in the case of salutaridine was a two-peaked distribution curve obtained (Fig. 2). The smaller peak was due to salutaridine itself; however, the peak R_t 18.5 min most probably has to be traced back to interference of thebaine with the salutaridine antibodies since thebaine was present in these fractions at *ca* > 100-fold higher concentration. This corresponded to a cross-reactivity of about 0.3–0.5% of thebaine in the salutaridine assay. Subjecting this HPLC-separated fraction to TLC and assaying the plate with the salutaridine specific RIA showed that only the thebaine area yielded immunopositive material. This makes it most likely that small amounts of salutaridine cannot reliably be measured in the presence of a large excess (> 100-fold) of thebaine. (*R*)- and (*S*)-reticuline were found only in the appropriate fractions and absolutely no interference with other materials was observed. Extract dilution curves were taken with the six alkaloid RIAs and were subsequently subjected to logit transformation. Comparison with the standard curves showed parallel displacement and yielded identical slopes. This again is proof that no grossly

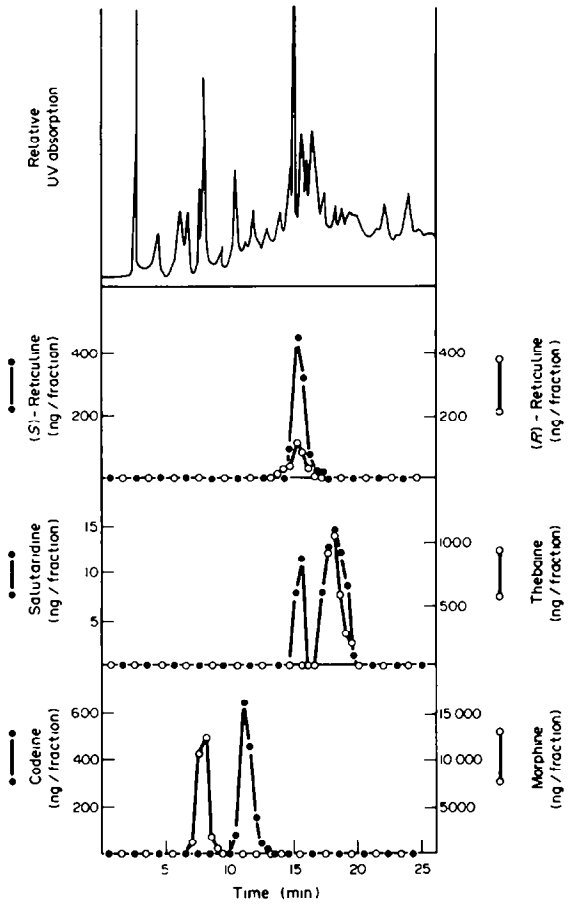

Fig. 2. HPLC fractionation of a crude alkaloid mixture derived from a mature *Papaver somniferum* plant. Each fraction (0.75 ml) was assayed after dilution by the six alkaloid RIAs. R_t for (*S*)- and (*R*)-reticuline 15.5 min, salutaridine 15.5 min, salutaridinol (I) 12.0 min, thebaine 18.5 min, codeine 8 min, morphine 11.5 min.

interfering substances were present in these extracts [11].

Distribution of opium alkaloids in herbarium samples

Having proven the specificity of the assay systems and shown that salutaridine cannot be determined in the presence of a 100-fold excess of thebaine, we analysed the occurrence of the six opium alkaloids in dried leaf tissue of *Papaver*. The genus *Papaver* has been the subject of many chemical investigations for the alkaloids thebaine, codeine and morphine [12]. Very little work has been published about the simultaneous occurrence of the six opium alkaloids for which RIA methods are now available. First, we turned to taxonomically authenticated herbarium material of the genus *Papaver* of which *ca* 150 species are known. We obtained and analysed 100 species. Being aware of the occurrence of 'chemical races' within one species [13], we attempted to analyse at least three samples of the same species collected from different geographical origins. About 3–10 mg samples of leaf tissue were removed from the herbarium samples, extracted and aliquots of the extracts were analysed with RIA. Under the conditions used, the detection limits for the alkaloids investigated here were 2×10^{-5}% (*S*)-reticuline, 7×10^{-5}% (*R*)-reticuline, 1×10^{-4}% salutaridine, 5×10^{-5}% thebaine and 3×10^{-5}% codeine and morphine, based in each case on the dry wt of the sample. Only in three *Papaver* species was not one of the six alkaloids in question detected. These were *P. arenarium* Bieb., *P. pavonicum* Fisch. & C. A. Mey. and *P. pulvinatum* A. Tolm. The (*S*)- and (*R*)-reticuline contents were found to be relatively low (in most cases $< 10^{-3}$%) but the alkaloids occurred in 97 of the 100 species tested. The crucial step in the biosynthesis of morphine alkaloids is the transformation of (*R*)-reticuline to salutaridine. Surprisingly this alkaloid was found to occur in appreciable amounts in as many as 35 species and in trace quantities in another 27. The highest content was found in a sample of *P. tauricola*. Less common was the occurrence of thebaine. It was found in 21 species; for 10 of which several geographical origins were investigated. Morphine and codeine have hitherto been observed only in *P. somniferum*, *P. setigerum* DC. and, as minor alkaloids, in *P. decaisnei* Hochst [14]. This investigation has proved their additional occurrence in *P. gracile* Boiss and *P. cylindricum* Cullen (see Table 3).

Apart from the species mentioned above, trace amounts of codeine and/or morphine were detected in samples of 15 other species. These, however, will have to be re-investigated using authenticated living material and HPLC prefractionation before application of RIA. The alkaloid patterns of some herbarium samples with the highest alkaloid contents are shown in Table 3.

It should be noted that the RIA method for the determination of the six opium alkaloids yielded a result comparable to that obtained from living material even with a 200-year-old herbarium specimen of *P. somniferum*. The surprising stability of these alkaloids in dried leaves is thus demonstrated.

From the above results it clearly follows that it is not the transformation of (*R*)-reticuline to salutaridine which is limited to only a few species, but the further metabolism of salutaridine to thebaine, and especially from there to codeine and morphine which is expressed in only very few cases.

Distribution of alkaloids in living plants

Undoubtedly *P. somniferum* is the species which contains the highest amount of codeine and morphine. Plants from 372 different geographical origins were therefore tested for their ability to produce high amounts of the six alkaloids. Five days after petal opening, samples of latex, leaf and shoot were taken and analysed for their alkaloid content. In Table 4, the maximal yields of the individual alkaloids in these samples are reported.

Contents as high as 4% morphine were found to occur in the shoot while 2.8% morphine and 0.6% codeine were found in the leaves of some plants. The predominance of morphine and thebaine over codeine is also reflected in the latex samples. Only the latex of one single plant out of the 315 tested showed a higher codeine than morphine concentration. In this case the absolute content of codeine (17 μg/μl) was, however, too small to be useful for breeding purposes. For all the 315 latex samples, the ratio (*S*):(*R*)-reticuline was determined. It was shown that this quotient was not constant in these plants but deviated from 0.4 to 62. A plot of the ratios revealed that most frequently values between 2.5–3.2 are found. This means that there was an about three-fold higher concentration of (*S*)- than of (*R*)-reticuline present in the latex. This

Table 3. Alkaloid distribution as determined by RIA in some *Papaver* species

			Alkaloid content ($\times 10^{-3}$% dry wt)					
Species	Author	Year and herbarium	(*S*)-Reticuline	(*R*)-Reticuline	Salutaridine	Thebaine	Codeine	Morphine
P. acrochaetum	Bornm.	1957/M*	28	42	330	1.4	0	0
P. caucasicum	Bieb.	1938/M	3.2	2.4	389	8.3	0	0
P. cylindricum	Cullen	/Lo	23	8	49	687	0.9	11
P. gracile	Boiss.	1983/M	5.1	3.9	2.0	3.6	14	64
P. persicum	Lindl.	1983/M	7.4	1.4	76	160	0	0
P. somniferum	L.	1976/M	55	183	25	12	43	895

*M, State Herbarium, Munich; Lo, Kew Gardens, London.

Table 4. Maximal yields of six opium alkaloids observed in different organs and latex of field-grown *Papaver somniferum* plants from 372 (latex 315) different geographical origins (triplicate samples)

	(*S*)-Reticuline	(*R*)-Reticuline	Salutaridine	Thebaine	Codeine	Morphine
Latex (as $\mu g/\mu l$)	68	13	17	200	79	430
Leaf (as % dry wt)	0.26	0.45	0.04	0.4	0.6	2.8
Shoot (as % dry wt)	0.29	0.05	0.04	0.76	1.4	3.9

predominance of the (*S*)-form holds true for over 90% of all the plants analysed. The ratios reported previously for opium and plant samples, (*S*):(*R*)-reticuline 1.5:1 [15] and 6:1 [16], demonstrate that these determinations are valid only for a specific plant sample taken at a specific developmental stage and cannot be generalized. The frequency distribution for codeine, which is pharmaceutically the most valuable compound, is shown in Fig. 3. The average codeine content in the leaf discs was 0.005% (based on dry wt). Plants which gave the highest values (leaf 0.4, shoot 1.4%) were reanalysed to eliminate errors which might have occurred when only three leaf discs per plant were analysed. Individual high codeine-yielding plants were selected and will be used for further breeding experiments.

Formation of opium alkaloids during the germination of P. somniferum

It has been established that during seed germination of *P. somniferum* reticuline as well as thebaine is formed at a very early stage and that the fifth day after germination represents a stage of intensive alkaloid formation [17]. These observations were recently corroborated for *P. bracteatum* [7], where it could be shown that seedlings were devoid of laticifer initials until 3 days after germination whereupon their numbers increased with time. Laticifers were first detected in radicles. The appearance of thebaine co-occurred with the onset of laticifer formation [7].

In order to gain insight into the biosynthesis of morphinane alkaloid formation, we applied the RIAs developed here to monitor the formation of individual opium alkaloids in germinating seeds. *P. somniferum* seeds

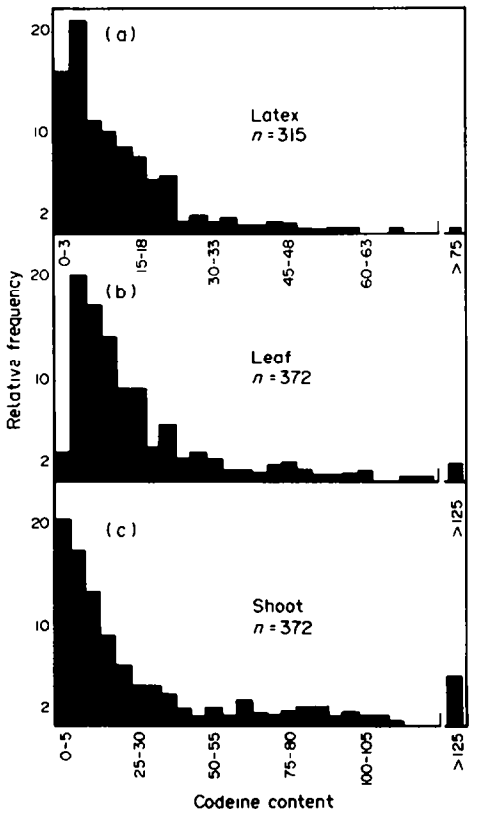

Fig. 3. Frequency distribution of the codeine concentration in latex ($\mu g/\mu l$), leaf and shoot samples of *P. somniferum* plants of different geographical origin (10^{-3}%).

were grown for a period of 8 days after which time the plants had reached a total length of *ca* 3.5 cm, three to four primary leaves and a 2–3 cm length root. The time course of alkaloid formation is shown in Fig. 4. All alkaloids were formed during this period of time. Both reticuline enantiomers as well as salutaridine and thebaine increased in parallel, starting 3 days after germination. Codeine was formed at a much slower rate and morphine was detected in traces only 6 days after germination. Thebaine was the major alkaloid, as noted previously for *P. bracteatum* [7]. Based on concentration per unit dry wt, it can clearly be seen (Fig. 4A) that the rate of alkaloid formation was not just a consequence of a gain in dry wt per plant (Fig. 4B).

Four days after seed germination, it was possible to dissect the seedlings into root, shoot axis and leaves. The time course of alkaloid formation was followed during a further 4-day period and the alkaloids were determined individually in each of the three organs. As seen in Fig. 5, again alkaloid synthesis proceeded rapidly on a dry wt basis. Quite clearly alkaloids were found in the highest concentration in the roots, except for codeine which was detected at maximal level in the shoot axis. The time course of alkaloid accumulation indicates that the most rapid increase in alkaloid content had taken place in the root where the dominant alkaloid, thebaine, was detected in larger concentrations in the shoot axis first and it was only after day 7 that the level in the root was higher than that in the shoot. The question remains open whether thebaine was formed first in the root and then transported to the shoot, or whether a transformation of a precursor-like salutaridine occurred in the shoot itself where it was subsequently stored. The low level of salutaridine found in the seedlings up to day 6 suggests that this compound is metabolized rapidly. The difference in the accumulation pattern between salutaridine and thebaine excludes the possibility that thebaine has disturbed the salutaridine determination by cross-reaction. Combination of radiotracer techniques and immunological determination of individual alkaloids will lead to more detailed knowledge of the formation and storage of alkaloids in *Papaver*.

As these few examples have shown, the large capacity of RIA (a trained technician can perform up to 500 quantitative analyses per day), its specificity and its sensitivity represent a major advantage over current analytical methods such as HPLC and TLC. With RIAs available for individual opium alkaloids, specific breeding programmes for high yielding thebaine, codeine or morphine strains become possible. It will also prove to be a valuable method in screening large numbers of cell cultures for *Papaver* alkaloids and will be found useful in the study of the biosynthetic pathway leading from reticuline to morphine.

EXPERIMENTAL

Mps are uncorr. ^{1}H NMR spectra were recorded at 200 MHz (TMS as internal standard). MS were measured at 70 eV. TLC, silica gel SIL G/UV$_{254}$ (Macherey and Nagel); prep. TLC, silica gel 60 F-254 (E. Merck). Immunoassay equipment consisted of semiautomatic pipetting stations and mixers (Analmatic) and a centrifuge (Heraeus). Radioactivity was determined in a liquid scintillation spectrometer with punched tape output. RIA calculations were done on a programmed off-line calculator using the spline approximation method [18]. HPLC was performed with a Nucleosil 10 C 18 column (0.4 × 25 cm) (Macherey and Nagel) using a linear gradient of 0.0085% H_3PO_4 (0 min) to MeCN–0.0085% H_3PO_4 = 1:1 (30 min). Flow rate was 1.5 ml/min; detection at 284 nm.

Plant material. Seeds were obtained from botanical institutions worldwide. A *P. somniferum* inbred line which produces large amounts of morphine under our climatic conditions was used. The plants were grown outdoors at a distance of 70 cm from plant to plant. For germination expts, 0.2 g of washed and sterilized seeds was grown in clear plastic Petri dishes (9 × 5 cm)

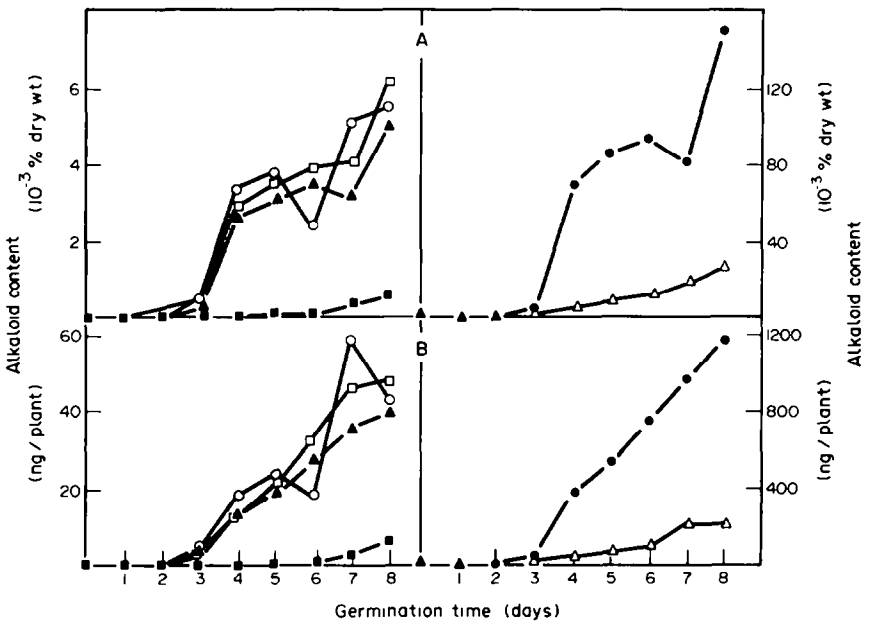

Fig. 4. Formation of (*S*)-reticuline (□), (*R*)-reticuline (○), salutaridine (▲), thebaine (●), codeine (△) and morphine (■) during an 8-day period of growth from seeds of *p. somniferum*.

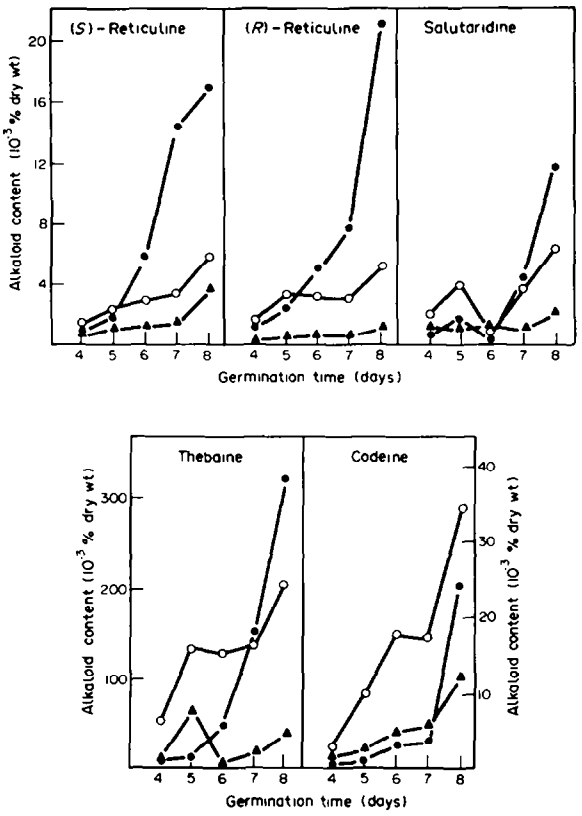

Fig. 5. Kinetics of the formation of (*S*)-reticuline, (*R*)-reticuline, salutaridine, thebaine and codeine in roots (●), shoot axis (○) and leaves (▲) of *P. somniferum* seedlings during the first 8 days of development.

containing 4 g vermiculite and 27 ml Knop's nutrient soln. Growth occurred under 1500 lx light (cool white fluorescent lamps). Herbarium material was obtained from the State Herbarium, Munich; University of Göttingen; Kew Gardens, London; Senckenberg Museum, Frankfurt; Botanical Garden, Geneva; University of Istanbul and University of Leningrad.

Extraction procedures. Small amounts (3–10 mg) of dried leaf material were removed from herbarium sheets. The material was extracted with 1.5 ml 80% MeOH in 1.5 ml Eppendorf vials for 12 hr at 60°. The alkaloids under investigation were stable under these conditions. Addition of acid to the extraction procedure had no measurable effect. For selection purposes, samples from living material were used 5 days after petal opening. Latex was exuded (1 μl) and diluted with 80% MeOH. Samples were punched out of the youngest fully expanded leaf (5 mm diameter, 0.64 mg dry wt) and shoot samples were taken below the oldest leaf of a plant (1 cm length, 0.5 cm width, 2 mm deep, 2.6 mg dry wt). All samples were extracted with 80% MeOH (1 ml) for 12 hr at 60°. For the HPLC investigation, a *ca* 90-day-old *P. somniferum* plant (106 g fr. wt) was cut into pieces and extracted with 1 l. hot 80% EtOH for 12 hr at 60°. The solvent was evaporated and the dry residue taken up in H_2O (100 ml) and acidified with HCl to pH 4. The aq. soln was washed with petrol. The aq. phase was applied to a column of XAD-2 (2 × 15 cm), washed extensively with H_2O and the alkaloids were eluted with MeOH. The concentrate (2 ml) was used for HPLC (50 μl).

Chemicals. Codeine and morphine were purchased from Merck; thebaine was a gift from Boehringer, Ingelheim. Benzylisoquinolines and codeinone were synthesized according to standard procedures. Optically pure (*S*)- and (*R*)-norreticuline were synthesized according to ref. [19], salutaridine and salutaridinol I and II ref. [10], northebaine and norcodeine ref. [20] and normorphine ref. [21].

Synthesis of N-2-*carboxyethyl derivatives.* From all the alkaloids, except salutaridine, the *N*-2-carboxyethyl derivatives were synthesized according to ref. [9] and used as haptens. The synthetic procedure is exemplified here for codeine. Norcodeine (200 mg, 0.7 mmol) was dissolved in EtOH (6 ml) and acrylic acid (300 mg, 4.2 mmol) added. The mixture was kept for 2 hr at 60°. The reaction product crystallized. It was then recrystallized from EtOH yielding 199 mg (79%) *N*-(2-carboxyethyl)-norcodeine, mp 177–178° (EtOH), MS *m/z*: 357 $[M]^+$. In a similar manner were obtained *N*-(2-carboxyethyl)-(*S*)-norreticuline (56%), mp 149–151° (MeOH–Et_2O) (found: C, 63.25; H, 6.61; N, 3.80; calc. for $C_{21}H_{25}NO_6 \cdot 2/3H_2O$: C, 63.15; H, 6.65; N, 3.51%); $[\alpha]_D^{22}$ + 72.5° (*c* 1.091; MeOH); *N*-(2-carboxyethyl)-(*R*)-norreticuline, (52%), mp 149–151° (MeOH–Et_2O) (found: C, 63.12; H, 6.49; N, 3.68; calc. for $C_{21}H_{25}NO_6 \cdot 2/3H_2O$: C, 63.15; H, 6.65; N, 3.51%); $[\alpha]_D^{22}$ − 72.6° (*c* 1.06; MeOH); *N*-(2-carboxyethyl)-northebaine (75%), MS *m/z*: 369 $[M]^+$.

Salutaridine-7-(O-carboxymethyl)oxime. Salutaridine (195 mg, 0.6 mmol) was dissolved in 20 ml MeOH and aminooxyacetic acid (218 mg, 2.4 mmol) added. The mixture was stirred for 24 hr at room temp. Precipitated material was filtered off, the filtrate concentrated to 3 ml and the reaction product purified by prep. TLC ($CHCl_3$–MeOH–NH_4OH (25%), 75:34:4.5). The substance with R_f 0.3 was eluted. Yield 170 mg (0.4 mmol, 67%), ^1H NMR (CD_3OD): δ2.83 (3H, *s*, NMe), 3.72/3H, *s*, OMe), 3.83 (3H, *s*, OMe), 4.56 (2H, *s*, OCH_2COOH), 6.65, 6.85 (2H, ABq, J_{AB} = 8 Hz), 7.06 (1H, *s*), 7.27 (1H, *s*). The identity of the product with the target compound was further shown by MS of the methylated derivative: MS *m/z* (rel. int.): 428 $[M]^+$ (100), 413 (73), 355 (47), 324 (47), 149 (50). ^1H NMR ($CDCl_3$): δ2.75 (3H, *s*, NMe), 3.75, 3.83, 3.86, 3.92 (each 3H, *s*, 3 × OMe, COOMe), 4.79 (2H, *s*, OCH_2COOMe), 6.58 (1H, *s*), 6.86 (2H, *s*), 7.14 (1H, *s*).

^3H-Labelled tracers. (*S*)- and (*R*)-[N-C^3H_3]reticuline were synthesized from the corresponding *nor*-compound using *S*-adenosyl-L-[C^3H_3]methionine and an *N*-methyltransferase from *Berberis* cell cultures as enzyme catalyst [22]. Sp. act. was 7.2 Ci/mmol for (*S*)-, 3.2 Ci/mmol for (*R*)-reticuline as determined by the self-displacement method [23]. Salutaridinols I and II were synthesized from salutaridine as follows.

Method A [10]. A soln of salutaridine (14 mg, 0.043 mmol) in dry EtOH (0.6 ml) was added to NaB^3H_4 (0.31 mg, 0.008 mmol, 100 mCi, sp. act. 12.4 Ci/mmol) contained in an ice-cooled ampoule. After stirring for 2 hr at 0° and another 2 hr at room temp., the solvent was evaporated in a stream of N_2. The residue was treated with H_2O (0.2 ml) and extracted with $CHCl_3$ (5 × 1 ml). After concn the combined extracts were subjected to TLC ($CHCl_3$) and the plate was scanned for radioactivity. Salutaridinol II (R_f = 0.10) and salutaridinol I (R_f = 0.23) were eluted with MeOH and chromatographed ($CHCl_3$–Me_2CO–Et_2NH, 5:4:1) to yield salutaridinol I (R_f 0.66, 1.3 mCi, sp. act. 3.1 Ci/mmol) and salutaridinol II (R_f 0.44, 1.2 mCi, sp. act. 3.1 Ci/mmol).

Method B. Salutaridine (24 mg, 0.073 mmol) was dissolved in MeOH (0.5 ml) followed by $CeCl_3 \cdot 7H_2O$ (57 mg, 0.153 mmol). This mixture was cooled with ice and transferred to a cooled ampoule containing NaB^3H_4 (250 mCi, sp. act. 10 Ci/mmol). After 2 min stirring at 0° and 5 min at room temp. most of the MeOH was evaporated and H_2O (1 ml) containing 2 N KOH (3 drops) was added. The mixture (pH 8–9) was extracted with EtOAc (3 × 2 ml) and the combined organic phases were dried (Na_2SO_4) and evaporated to give a mixture of crude salutaridinols I and II (17 mg, 71%) containing traces of starting

material. Separation on a silica gel plate (0.25 mm, $CHCl_3$–MeOH–Et_2NH, 5:4:1) yielded salutaridinol I (5.0 mg, 18.2 mCi, sp. act. 1.18 Ci/mmol) as a white crystalline solid and salutaridinol II (11.5 mg, 40.9 mCi, sp. act. 1.17 Ci/mmol) as a colourless oil.

Salutaridinol I was used as tracer in the RIA of salutaridine. [7-^{3}H]Thebaine was synthesized by scale-down of the procedure given in ref. [10]. [7-^{3}H]Salutaridinol II (170 μCi; 3.1 Ci/mol) was treated at room temp. for 20 min with 0.5 N HCl (1.2 ml). 1 N NaOH (1 ml) was added and the labelled product extracted × 3 with 5 ml $CHCl_3$–*i*-PrOH (3:1, 3 × 5 ml). The organic phase was evaporated and the labelled material subjected to TLC (toluene–EtOAc–Et_2NH, 7:2:1; thebaine R_f 0.68; salutaridinol II $R_f = 0.24$) whereupon 80% of the radioactivity resided in the thebaine zone. The product was further purified in three additional solvent systems and proved to be free of any other labelled contaminant.

[6-^{3}H]Codeine was synthesized according to ref. [24]. Codeinone (4.4 mg, 15 μmol) was dissolved in EtOH (0.6 ml) and added to a suspension of NaB^3H_4 (1.9 μmol; 77.8 Ci/mmol) in EtOH (0.15 ml). After 24 hr at room temp., the material was chromatographed in $CHCl_3$–Me_2CO–Et_2NH (5:4:1). The zone with R_f 0.5 contained 95% of the labelled organic material. This material was purified by TLC in three more solvent systems in which it co-migrated in every case with codeine. The sp. act. was 20 Ci/mmol as determined by the self-displacement method [23]. [1-^{3}H]Morphine (24 Ci/mmol) was purchased from Amersham.

Coupling procedures to BSA. The *N*-2-carboxyethyl derivatives of the alkaloids and the salutaridine derivative were coupled to BSA (Behringwerke) according to the general protocol given here for codeine. A soln of (*N*-2-carboxyethyl)norcodeine (200 μmol) and BSA (180 mg, 3 μmol) in H_2O (10 ml) was adjusted with 0.1 M $NaHCO_3$ soln to pH 7.7. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide · HCl (948 mg, 4800 μmol) was added and the mixture stirred for 18 hr at room temp. The mixture was subsequently dialysed for 4 days at 4° against deionized H_2O and then lyophilized. Usually 180 mg of dried conjugate was obtained.

Immunization and antiserum production. The conjugate preparations were administered to 12- to 16-week-old rabbits as a 1:1 emulsion in Freund's complete adjuvant. After 4 weekly intradermal immunizations, intramuscular booster injections were given monthly and blood was collected 2 weeks after each booster. The collected sera were stored at −18° and found to be stable for several years under the conditions employed.

Performance of RIA. Details of the procedure have been given earlier [1, 9]. In brief, the assay was carried out as follows: incubation tubes received Pi-buffer saline (0.5 ml), pH 7.4 (PBS, 0.01 M phosphate, 0.15 M NaCl), dilute BSA (0.1 ml), dilute tracer (0.1 ml) (5–10 000 cpm) and standard or sample (0.1 ml). After mixing, dilute antiserum (0.1 ml) was added, the tubes were mixed again and incubated for 1 hr at room temp. followed by the addition of $(NH_4)_2SO_4$ (10 vol. saturated soln plus 1 vol. H_2O; 1 ml, to prevent crystallization during pipetting). After mixing, the tubes were incubated for 30 min at room temp. and then centrifuged. The pellets were washed once with 1 ml half-saturated $(NH_4)_2SO_4$ dissolved in H_2O (0.2 ml) and mixed with scintillation fluid (1 ml Minisolve, Zinsser). After thorough mixing, the tubes were counted for radioactivity. Under the conditions applied, the counting efficiency was 14%. Cross-reactions were determined according to ref. [25]. All RIAs were performed in triplicate.

Acknowledgements—We are grateful to Prof. W. Steglich, Bonn, for a gift of dihydrothebaine-ϕ, and to Prof. Brochmann-Hanssen, Berkeley, for a sample of oripavine. Our sincere thanks are due to the Herbaria mentioned, for allowing us to remove small samples of plant material. This investigation was supported by SFB 145 of Deutsche Forschungsgemeinschaft, Bonn, and by Fonds der Chemischen Industrie. The linguistic help of Dr. T. M. Kutchan in the preparation of this manuscript is gratefully acknowledged.

REFERENCES

1. Weiler, E. W. and Zenk, M. H. (1976) *Phytochemistry* **15**, 1537.
2. Weiler, E. W. (1983) *Biochem. Soc. Trans.* **11**, 485.
3. Farnsworth, N. R. and Morris, R. W. (1976) *Am. J. Pharmacol.* **148**, 46.
4. Lübke, K. and Nieuweboer, B. (1978) *Immunologische Tests für Niedermolekulare Wirkstoffe*. Thieme, Stuttgart.
5. Hodges, C. C. and Rapoport, H. (1982) *J. Nat. Prod.* **45**, 481.
6. Yoshikawa, T. and Furuya, T. (1985) *Planta Med.* **50**, 110.
7. Rush, M. D., Kutchan, T. M. and Coscia, C. Y. (1985) *Plant Cell Rep.* **4**, 237.
8. Hsu, A.-F., Brower, D., Etskovitz, R. B., Chen, P. K. and Bills, D. D. (1983) *Phytochemistry* **22**, 1665.
9. Weiler, E. W., Stöckigt, J. and Zenk, M. H. (1981) *Phytochemistry* **20**, 2009.
10. Barton, D. H. R., Kirby, G. W., Steglich, W., Thomas, G. M., Battersby, A. R., Dobson, T. A. and Ramuz, H. (1965) *J. Chem. Soc.* 2423.
11. Cekan, Z. (1975) *J. Steroid Biochem.* **6**, 271.
12. Stermitz, F. R. (1968) *Recent Adv. Phytochem.* **1**, 161.
13. Phillipson, J. D. (1983) *Planta Med.* **48**, 187.
14. Slavik, J. (1980) *Collect. Czech. Chem. Commun.* **45**, 2706.
15. Brochmann-Hanssen, E. and Nielsen, B. (1965) *Tetrahedron Letters* 1271.
16. Battersby, A. R., Evans, G. W., Martin, R. O., Warren, M. E. and Rapoport, H. (1965) *Tetrahedron Letters* 1275.
17. Martin, R. O., Warren, M. E. and Rapoport, H. (1964) *J. Am. Chem. Soc.* **86**, 4726.
18. Marschner, I., Dobry, H., Erhardt, F., Landersdorfer, T., Popp, B., Ringel, C. and Scriba, P. C. (1974) *Ärztl. Lab.* **20**, 184.
19. Rice, K. C. and Brossi, A. (1980) *J. Org. Chem.* **45**, 592.
20. Eli-Lilly Co. (1966) Dutch Patent Appl. 6, 515, 815.
21. Abdel-Monem, M. M. and Portoghese, P. S. (1972) *J. Med. Chem.* **15**, 208.
22. Wat, C.-K., Steffens, P. and Zenk, M. H. (1986) *Z. Naturforsch.* **41c**, 126.
23. Chervu, L. R. and Murty, D. R. K. (1975) in *Radioimmunoassay* (Freeman, L. M. and Blaufox, M. D., eds) p. 33. Grune and Stratton Press, New York.
24. Gates, M. (1953) *J. Am. Chem. Soc.* **75**, 4340.
25. Abraham, G. E. (1975) *J. Steroid Biochem.* **6**, 261.