not mediated by known detoxification mechanisms [2]; (ii) the activity is not affected by the multidrug resistance family of proteins with a molecular weight of approximately 170,000 [5]; and (iii) formation of cytotoxic metabolites of FUra such as FdUMP was at least not decreased upon *ras* transformation since cytotoxicity of FUra was the same or even not significantly increased in c-H-*ras* transformed and control cells. It is not yet known whether this newly demonstrated dFUrd activity enhancement can be exploited optimally in c-H-*ras* transformed cells.

It is frequently stated that continuous dFUrd treatment might be more effective than bolus dFUrd [6]. Nude mice bearing NIH 3T3 cells transformed with *ras* and other oncogenes as discussed above offer an interesting model to define optimal treatment with dFUrd. This is especially true for colorectal tumours since *ras* involvement was frequently demonstrated in this tumour type [3] and dFUrd has proven efficacy in the treatment of this tumour. Since activated *ras* oncogenes have also been associated with resistance to cisplatin and to ionizing radiation [4, 10], the present data add to the understanding of the rather unique efficacy of fluoropyrimidines in the treatment of colorectal cancer.

In summary, transformation of NIH 3T3 cells with c-H-*ras* has been demonstrated to result in significantly increased activation of 5'-deoxy-5-fluorouridine and significantly increased cytotoxicity *in vitro* as compared to non-transformed NIH 3T3. FUra cytotoxicity appeared to be increased also *in vitro* upon transformation; the level of significance however was beyond that of accepted significance ($0.05 < P < 0.01$). Furthermore dFUrd proved to be less active *in vivo* in nude mice bearing v-*fos* transformed NIH 3T3 cells than in nude mice bearing c-H-*ras* transformed cells.

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# Hyposmolarity-sensitive release of taurine and free amino acids from human lymphocytes

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The ability of human lymphocytes to regulate their volume in anisosmotic conditions is well documented [for recent reviews see Refs. 1 and 2]. When exposed to hyposmotic media, human lymphocytes show rapid initial swelling followed by a regulatory phase in which cells return to near normal volume. The regulatory volume decrease following osmotic swelling results from the loss of intracellular osmotically active solutes, mainly $K^+$ and $Cl^-$. In human lymphocytes these ionic regulatory fluxes occur through separate $K^+$ and $Cl^-$ pathways [1, 3, 4], whereas in other cells they are carried by $K^+/Cl^-$ cotransport systems, activated by the osmotic stress [5].

Although the reduction in the amount of intracellular solutes leading to cell volume regulation corresponds largely to the loss of $K^+$ and $Cl^-$, some amino compounds also behave as intracellular osmolytes and may contribute at some extent to the regulatory process. The involvement of free amino acids (FAA) in osmoregulation in aquatic vertebrates and invertebrates naturally exposed to fluctuations in external osmolarity is well recognized [6, 7].

Less information exists about a similar role for FAA in mammalian cells although it is known that a number of these cells possess mechanisms for cell volume adjustment. FAA, notably taurine, are released from Ehrlich ascites cells [8], superfused rat brain [9, 10] and cultured astrocytes [11] upon hyposmotic stimulation. Human lymphocytes contain high concentrations of taurine, around 35 mM, and other FAA [12, 13], which may contribute as volume regulatory osmolytes. In the present work, changes in FAA content of human lymphocytes in response to hyposmotic stress were examined in order to evaluate its involvement in the mechanisms of cell volume adjustment. Properties of the release of [$^3$H]taurine were also examined to obtain insight into the mechanism of the volume-sensitive release of FAA in human lymphocytes.

*Methods*

*Lymphocyte isolation.* Blood was collected from a constant group of human donors, in sterile syringes containing EDTA (10%), and the blood sample was diluted 1:1 with Krebs–bicarbonate medium (KBM) containing (in mM): 118 NaCl, 4.7 KCl, 1.1 $KH_2PO_4$, 1.0 $CaCl_2$, 1.2 $MgSO_4$, 25 $NaHCO_3$ and 5 glucose, pH 7.4, adjusted by bubbling with $O_2/CO_2$ (95%/5%). Cells were isolated by the procedure of Boyum [14] as follows: 4 mL of diluted blood sample was layered carefully on 3 mL of Hystopaque (Sigma) in 15-mL conical centrifuge plastic tubes, and centrifuged at 400 *g* for 40 min. The interface containing lymphocytes was separated and washed once with KBM and the pellets were resuspended in KBM. The suspension obtained contained negligible contamination of platelets and polymorphonuclear cells and was free of erythrocytes. Cell viability, estimated by trypan blue exclusion, was about 98%.

*Release of* [$^3$H]*taurine.* Cells were preincubated in KBM containing [$^3$H]taurine (5 μM final concentration) at 37° for 1 hr. After this loading period, cells were filtered in Millipore filters (0.65 μM pore). For the time–course experiments, filters were transferred to glass superfusion chambers of 0.25 mL and superfused at a flow rate of 0.8 mL/min with KBM at 37°. Fractions of the perfusate were collected at 1-min intervals directly into scintillation vials. After a washing period of 8 min, the baseline efflux was attained and cells were then stimulated for 8 min with media of reduced osmolarity. At the end of the superfusion, radioactivity remaining in cells and that of collected samples was measured by scintillation spectrometry. In other experiments, filters were transferred to vials containing 1 mL of the different experimental media and incubated during 8 min at 37°. At the end of this incubation period, radioactivity was measured in incubation media and filters. Results are expressed as fractional release, i.e. the radioactivity in fractions as percent of total radioactivity in the cells at the start of superfusion or incubation, excluding the washing period. The drugs to be tested were added 15 min before the end of the loading period and were present during all the superfusion or incubation periods. When drugs were dissolved in solvents other than water, controls were exposed to the same concentration of the solvent used.

*Determination of endogenous free amino acids.* Free amino acids were extracted with 70% ethanol and derivatized with *O*-phthaldialdehyde. The amino acid content was determined by reversed phase HPLC in a Beckman chromatographic system, equipped with an Ultrasphere column.

*Results and Discussion*

The concentration of FAA of human lymphocytes is shown in Table 1. Taurine, with a concentration of 239 nmol/mg protein was the most abundant FAA, confirming previous observations by Fukuda *et al.* [13]. Taurine accounted for more than 65% of the total FAA pool. Other

Table 1. Free amino acid content of human lymphocytes

| Amino acid | nmol/mg protein |
|---|---|
| Glutamic acid | 44.5 ± 0.40 |
| Histidine | 7.8 ± 0.69 |
| Glycine | 19.5 ± 1.5 |
| Taurine | 238.7 ± 7.3 |
| β-Alanine | 26.2 ± 1.6 |
| Alanine | 20.2 ± 2.1 |
| Serine | 8.3 ± 0.5 |
| Valine | 2.8 ± 0.19 |
| Leucine | 1.8 ± 0.09 |
| Isoleucine | 2.1 ± 0.09 |

FAA content was determined in ethanol extracts by reversed phase HPLC. Results are means ± SE of 6 separate determinations.

FAA present in lymphocytes included glutamic acid, at a concentration of 44.5 nmol/mg protein, glycine, alanine and β-alanine at concentrations ranging from 20 to 26 nmol/mg protein (Table 1). The cellular volume/mg protein was calculated to be 8.17 μL, considering an average cell volume of 190 $\mu m^3$ ($0.19 \times 10^{-6}$ μL) [2, 15] and $43 \times 10^6$ cells/mg protein. Accordingly, the concentration of taurine was found to be 27.5 mM, which is within the range reported [12], and that of total FAA was 42.7 mM. Those values may be even higher, since the osmotically active water in human lymphocytes corresponds to only 68% of the cellular volume [3].

Upon incubation in media of decreasing osmolarity, the FAA content of cells decreased. Most FAA responded to hyposmolarity, but differences were observed in the amount released by cells and in the sensitivity to the stimulus. According to these differences, FAA may be grouped as follows: (1) Taurine, glutamate and histidine were tightly retained by the cells in isosmotic conditions but were released in response to reductions in osmolarity, even to small decreases (Fig. 1A). The cellular content of these amino acids decreased 70–90% by reducing osmolarity to 150 mOsmol (Fig. 1A). The largest decrease (almost 90%) was observed for taurine. (2) Glycine, alanine and β-alanine were also strongly retained by cells in isosmotic conditions but upon stimulation with hyposmolar medium (150 mOsmol), cells still retained 35–40% of their initial content (Fig. 1B). (3) Valine, isoleucine and serine were released in isosmotic conditions and were practically unresponsive to hyposmolarity (Fig. 1C). Reduction in the intracellular content of FAA was due to an activation of efflux since the decrease in cellular content was quantitatively accounted for by the concentration found in the medium (Fig. 1).

The time–course of taurine release in response to hyposmolarity was examined by following the efflux of [$^3$H]taurine in hyposmotically stressed cells. Figure 2 shows that decreasing osmolarity to 150 mOsmol resulted in a rapid release of [$^3$H]taurine which attained a maximum within the first minute (after subtracting the dead space of the superfusion system) and then declined despite the persistence of the stimulus. Reduction in osmolarity was obtained by decreasing the $Na^+$ concentration of the medium, but it is the hyposmolar condition and not the reduction of $Na^+$-concentration which induces taurine release, since a solution with low $Na^+$ but made isosmotic with sucrose did not elicit the release of [$^3$H]taurine.

The hyposmolarity-sensitive release of [$^3$H]taurine was unaffected by decreasing the temperature to 15°, but it was reduced by 60% at 4°. This observation suggests that the release of taurine is not energy dependent. The inhibition observed at low temperature may be due to changes in membrane fluidity that may affect diffusional processes.

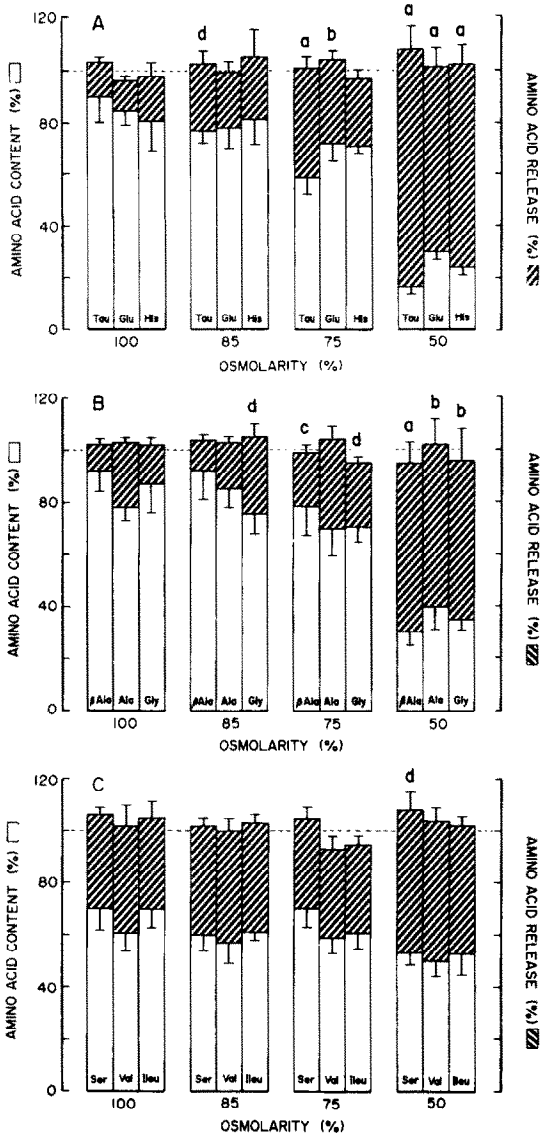


Fig. 1. Effect of decreased osmolarity on FAA content in human lymphocytes. Cells were incubated in isosmotic medium (300 mOsmol) or in media of decreased osmolarity: 0.85, 0.7 or 0.5 of the isosmotic medium, during 8 min. After incubation, cells were centrifuged and washed, and FAA were extracted with 70% ethanol. FAA content in cell extracts (white bars) or in incubation media (dashed bars) was measured by reversed phase HPLC. Results are expressed as percent change in cells or in incubation media, with respect to the endogenous FAA content in non-incubated cells (100%, broken line). (A) Changes in taurine, glutamate and histidine. (B) Changes in β-alanine, alanine and glycine. (C) Changes in valine, serine and isoleucine. Results are the means ± SE of 4–6 experiments. FAA release in hyposmotic media was significantly different from isosmotic medium by: $^{a}P < 0.001$; $^{b}P < 0.01$; $^{c}P < 0.02$; and $^{d}P < 0.05$.

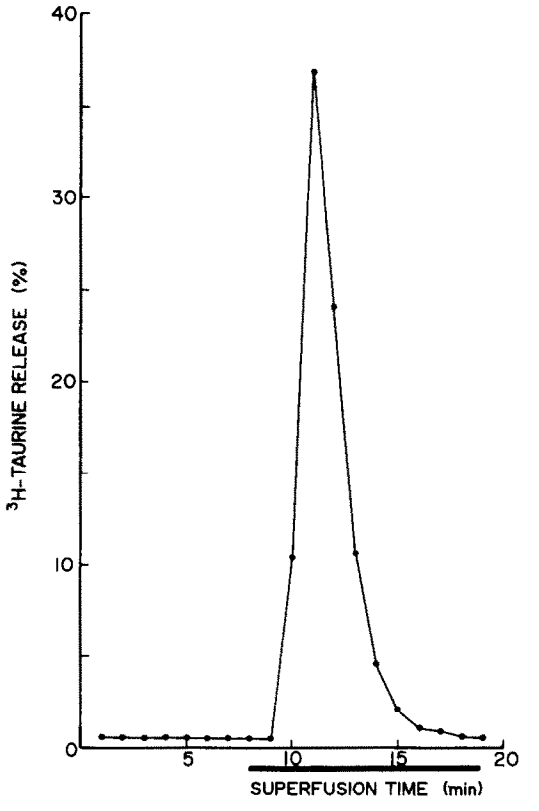


Fig. 2. Time–course of [$^3$H]taurine release stimulated by decreased osmolarity. Loading and release conditions were as described in Methods. During the time indicated by the bar, the superfusion medium (isomotic) was replaced by a hyposmotic medium (150 mOsmol, 35 mM NaCl). Results are expressed as fractional release as defined in Methods. The graph corresponds to a representative experiment from a total of 4.

Volume regulation in human lymphocytes is associated with losses of cellular $K^+$ and $Cl^-$ [4, 5]. The change in $K^+$ efflux activated by hyposmolarity seems to be conductive at least in part [4, 5]. $K^+$ fluxes associated with cell swelling are inhibited by antagonists of $Ca^{2+}$-dependent $K^+$ channels such as quinine, tetraethylammonium and 4-aminopyridine, and by agents interfering with the $Ca^{2+}$-calmodulin system like chlorpromazine and trifluoperazine [1, 3, 4]. The volume-induced increase in $Cl^-$ efflux occurs simultaneously but independently of the $K^+$ fluxes. $Cl^-$ conductance associated with volume changes in human lymphocytes is blocked by dipyridamol and by the disulfonic stilbene derivatives 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) [4]. To investigate whether the hyposmolarity-induced release of FAA was associated with the ionic fluxes activated by swelling, the effects of quinidine, barium, tetraethylammonium (TEA) and gadolinium on the release of [$^3$H]taurine were examined. The concentrations used were those reported to inhibit ionic fluxes [1, 3, 4], i.e. 75 μM quinidine, 15 mM barium, and 15 mM tetraethylammonium. Gadolinium was used at concentrations of 10 and 100 μM. None of these compounds modified taurine efflux (Table 2). Higher concentrations, particularly those of quinidine, were toxic to cells. DIDS showed an inhibitory effect on the hyposmolarity-sensitive release of taurine. The effect of DIDS was examined on the release of labeled as well as of endogenous taurine due to some quenching produced by DIDS at high concentrations in the experimental procedure used for measuring radioactivity. The inhibitory effect of DIDS was concentration dependent, with a maximal inhibition of about 40% at 200 μM (Fig. 3). Inhibition close to

Table 2. Effect of omission of $Na^+$ or $Cl^-$ and of inhibitors of ionic fluxes on the release of [$^3$H]taurine evoked by hyposmolarity

| Conditions | [$^3$H]Taurine release (%) Isosmotic | Hyposmotic |
|---|---|---|
| Control | 6.1 ± 0.8 | 72.7 ± 6.2 |
| $Na^+$-free | 5.9 ± 0.3 | 71.0 ± 5.8 |
| $Cl^-$-free | 5.0 ± 0.1 | 67.9 ± 1.7 |
| TEA, 15 mM | 6.4 ± 0.3 | 69.7 ± 5.9 |
| Barium, 15 mM | 7.0 ± 0.8 | 71.0 ± 3.5 |
| Quinidine, 75 μM | 5.1 ± 0.9 | 73.7 ± 2.1 |
| Gadolinium, 10 μM | 6.8 ± 0.7 | 71.7 ± 2.9 |

Cells were loaded with [$^3$H]taurine and incubated as described in Methods, with isosmotic medium or with a medium of reduced osmolarity (150 mOsmol). The ionic composition was modified in both, isosmotic and hyposmotic media. $Na^+$ and $Cl^-$ in the experimental solutions were replaced by the corresponding salts of choline and gluconate. Drugs were present during the last 15 min of the loading period and in media during all incubation periods. When solvents other than water were used, controls were exposed to the same amount of solvent. Results are expressed as fractional release (%) and are the means ± SE of 4–12 experiments.

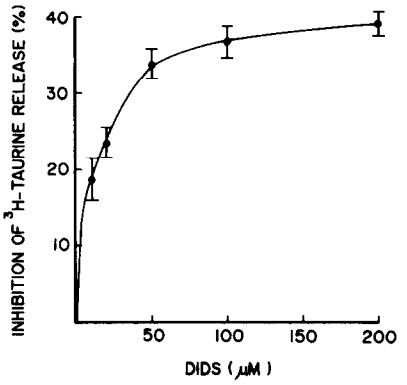


Fig. 3. Effect of DIDS on the hyposmolarity-sensitive release of endogenous taurine. Lymphocytes obtained as described in Methods were incubated in isosmotic medium containing the concentration of DIDS indicated for each point for 30 min. After this time, cells were incubated for 8 min in hyposmotic medium (0.75 osmolarity) containing the same concentration of DIDS as in the isosmotic medium. Controls were incubated in isosmotic and hyposmotic media without DIDS. Taurine released to the incubation medium and that remaining in cells at the end of the experiment was measured by HPLC. Results are expressed as percent inhibition of taurine released by hyposmotic medium in the absence of DIDS. Results are mean ± SE of 3–8 experiments.

maximal was observed in the presence of 50–100 μM DIDS. A noticeable inhibition was observed at 10 μM DIDS (Fig. 3). The inhibition of DIDS increased in a $Cl^-$-free medium. No effect on the release of [$^3$H] taurine was observed in the presence of pimozide (10 μM) or trifluoperazine (10 μM) (results not shown). All these observations suggest that the hyposmolarity-sensitive release of FAA occurs independently of the ionic fluxes activated during the volume regulatory process and of $Ca^{2+}$-mediated transduction reactions. In further support to this notion is the observation that activating $K^+$-fluxes in isosmotic conditions by A23187 did not elicit [$^3$H]taurine release (results not shown). It has been observed in human lymphocytes that $K^+$-fluxes in isotonic conditions are activated in the presence of the ionophore [3].

The mechanism responsible for the release of FAA in response to hyposmolarity is unclear at present. The hyposmolarity-sensitive efflux may result from a stimulation of the $Na^+$-dependent, carrier-mediated transport system for amino acids or from an activaton of leak pathways. To investigate whether taurine efflux might occur through the carrier transport system working outwards, the effect of removal of external $Na^+$ was examined on the spontaneous and the hyposmolarity-sensitive release of taurine. Under these conditions a $Na^+$ gradient inside > outside is imposed, which should drive taurine efflux in that direction. Also, evidence of the ability of the carrier to operate transporting intracellular taurine to the extracellular space was tested by measuring in an isosmotic medium the release of [$^3$H]taurine upon increasing the concentration of extracellular unlabeled taurine. Results of these experiments showed that neither the spontaneous nor the hyposmolarity-sensitive release of [$^3$H]taurine were affected by $Na^+$ omission (Table 2). Also, the efflux of previously accumulated [$^3$H]taurine was not activated by homoexchange (results not shown). These results argue against the involvement of the $Na^+$-dependent carrier on the swelling-associated release of taurine. Moreover, the insensitivity of taurine release to decreases in temperature supports this conclusion since the temperature-dependence of the taurine carrier is well established [16].

Results of the present study indirectly suggest that FAA, particularly taurine, participate in volume regulatory processes in human lymphocytes. As compared to ionic osmolytes, the contribution of FAA may be relatively small, since 60%, on the average, of a total FAA pool of about 43 mM leaves the cell in response to large decreases in osmolarity (150 mOsmol). Lymphocytes, as many other cells, have an anionic deficit with respect to cation concentration. The potassium concentration in human lymphocytes is about 140–170 mM [3, 17], whereas the $Cl^-$ content of cells is much lower. There are large discrepancies in the values reported for intracellular $Cl^-$ in lymphocytes, varying from 30 to 90 mM [3, 18, 19]. Moreover, the free cytoplasmic concentration of $Cl^-$ may be significantly different from the total cellular concentration reported, since compartmentation is suggested by the several components observed in the kinetics of $Cl^-$ efflux [19]. These observations suggest that not all $Cl^-$ in the cell may be available for volume regulation. In these conditions the contribution of non-electrolyte solutes, especially FAA, may be important to compensate for the ionic deficit.

In summary, this study has shown that human lymphocytes possess a hyposmolarity-sensitive release of FAA, particularly of the most abundant one, taurine. This release may contribute to the volume regulatory decrease in lymphocytes.

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# Cholesterylsuccinyl-*N*-hydroxysuccinimide as a cross-linking agent for the attachment of protein to liposomes

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Immunoglobulin-coated liposomes containing drugs have been examined (i) for their ability to specifically interact with cognate surface antigen on tumour cells and (ii) for targeting to tumours in animals [1]. Antibody coated liposomes have been prepared by Hashimoto *et al.* [1] who incorporated *N*-(*m*-maleimido benzoyl) dipalmitoyl-phosphatidyl ethanolamine into liposomes which were then reacted with immunoglobulin. Covalent attachment occurs via protein sulphydryl-SH groups to the maleimido area of the modified phospholipid. Attachment of immunoglobulins has also been achieved through a disulphide sulphydryl group exchange reaction using liposomes containing dipalmitoyl phosphatidyl-ethanolamine-3-(2-pyridyldithio) propionate [2]. Less-specific cross-linking procedures have made use of the coupling reagents toluene-2,4-diisocyanate and 1-ethyl-3-(dimethylaminopropyl) carbodiimide [3, 4].

In the present communication we describe an alternative procedure for attaching proteins and amino group-containing molecules to liposomes. The method makes use of cholesteryl-succinyl-*N*-hydroxysuccinimide incorporated into membrane structures of liposomes (Fig. 1). The resulting liposomes were found to be capable of interacting with amino group-containing substances to give molecues covalently attached to the surface.

## *Materials and Methods*

*Chemicals.* Cholesteryl hemisuccinate, *N*-hydroxysuccinimide and *N,N'*-dicyclohexyl carbodiimide were purchased from the Sigma Chemical Co., Pool, U.K.). [$^3$H]Puromycin (5 Ci/mmol) was supplied by Amersham (Bucks, U.K.). All other reagents were of analytical grade.

Preparation of cholesteryl hemisuccinyl-*N*-hydroxysuccinimide (Fig. 1). Cholesteryl hemisuccinate (97.4 mg, 0.2 mmol) and *N*-hydroxysuccinimide (25.3 mg, 0.02 mmol) were dissolved in 1 mL of dioxane. To this solution was added *N,N'*-dicyclohexyl carbodiimide (41.3 mg, 0.2 mmol) dissolved in 0.3 mL dioxane. The reaction mixture was allowed to stand at room temperature overnight. Dicyclohexylurea was removed by filtration and the clear filtrate taken to dryness at 37° under vacuum. The residue was taken up in 2 mL dioxane and allowed to stand at room temperature for 2 hr until no further crystallization of dicyclohexlurea occurred. Following filtration the final solution was concentrated to dryness and the residue recrystallized from isopropanol. m.p. 151–152°. Chromatography on silica gel $60F_{254}$ TLC plates developed in $CHCl_3$:methanol (9:1, v/v) gave a single spot (hydroxylamine/$FeCl_3$ for active ester and 1% $HClO_4$ with