After 4 days, most of the cells remained on the surface of the solid medium as spherical clusters of about 20–50 cells, but some were found to have migrated through the medium, appearing as flattened individuals on the floor of the dish. In subsequent experiments, therefore, the cell suspension was inoculated directly on to the floor of the dish, after cutting a central 5 mm well through the whole thickness of the medium. After incubation for 14 days these cells had proliferated centrifugally beneath the gel to produce a circular monolayer about 1 cm in diameter clearly visible to the naked eye (Figure 1). Subcultures into fluid medium showed that the cells survived in these conditions up to 6 weeks after inoculation, without any further addition of medium. This long survival suggests applications as a transport medium.

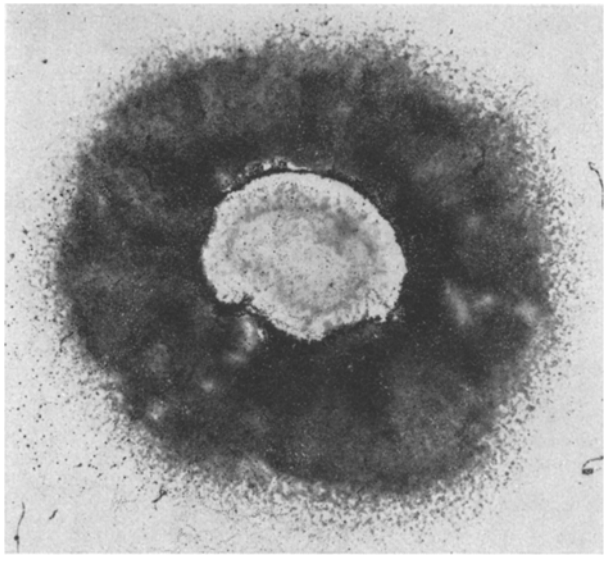

Fig. 2. Pattern of distribution of 3T6 fibroblasts after 14 days incubation. Haematoxylin and eosin. ×7.

The experiment was then repeated using smooth muscle cells, cultured in vitro from explants of pig aortic media. These cells did not survive, so the experiments were again repeated using, instead of agar, 3% agarose (initially agarose F.3083, later Indubiose A.45, L'Industrie Biologique Francaise, S.A.). This change was suggested by CUTLER's use of agarose-containing media[3], in a very similar system, for the detection of neutrophil chemotaxis. In agarose medium, the smooth muscle cells flattened on the floor of the dish and survived for up to 3 weeks beneath the medium close to the central well; the rate of cell proliferation was much less than that of the 3T6 fibroblasts, but for the present purpose that is of no consequence.

Experiments are now in progress in which materials are introduced into shallow wells in the periphery of the medium. These experiments suggest that the distribution pattern of the cells can be modified by this means; and therefore that the technique is suitable for the detection of both chemotaxis and growth-promoting substances (GOLD, TAYLOR, LEVENE and MITCHINSON, unpublished results).

An experiment can be concluded at any convenient time by fixing the adherent cells in situ by the addition of fixatives such as formol saline and then tipping the solid disc of medium out of the plate. The cells can then be stained by various routine methods (e.g. haematoxylin and eosin) but, because xylene dissolves the plastic, araldite should be used as the mountant. In this way a permanent record of the growth pattern is obtained (Figure 2). We believe the technique has broad applications.

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Blood Plasma Investigations by Resonance Raman Spectroscopy: Detection of Carotenoid Pigments

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Summary. Using Raman spectroscopy, we demonstrate that low levels of β-carotene, lycopene, and xanthophyll give rise to resonance enhanced bands in blood plasma. These results explain the significance of previously unidentified spectral maxima which have been related to the state of health of the blood donor.

In 1974 LARSSON and HELLGREN[1] reported that both the Raman and fluorescence spectra of human blood plasma change markedly depending on the state of health of the individual. The laser Raman spectrum of blood plasma exhibits three scattering maxima superimposed on a fluorescence background. We now report that these previously unidentified bands[1] arise from the carotenoids in blood plasma, and thus report the direct detection of carotenoids in blood plasma by resonance Raman spectroscopy.

Carotenoid pigments are intensely colored, and through the coupling of electronic and vibrational modes give rise to a resonance enhanced spectrum which can be detected at 10^{-7} M[2,3]. Previous work has shown that carotenoids are carried by plasma proteins at levels of ca. 10^{-6} M[4]. Thus, we believed that direct observation of carotenoids by the resonance Raman technique should be possible. Repetitive measurements on human blood plasma showed the three maxima at 1517, 1157 and 1005 cm^{-1} (Figure a, Ar^+ laser at 514.5 nm). These frequencies and relative

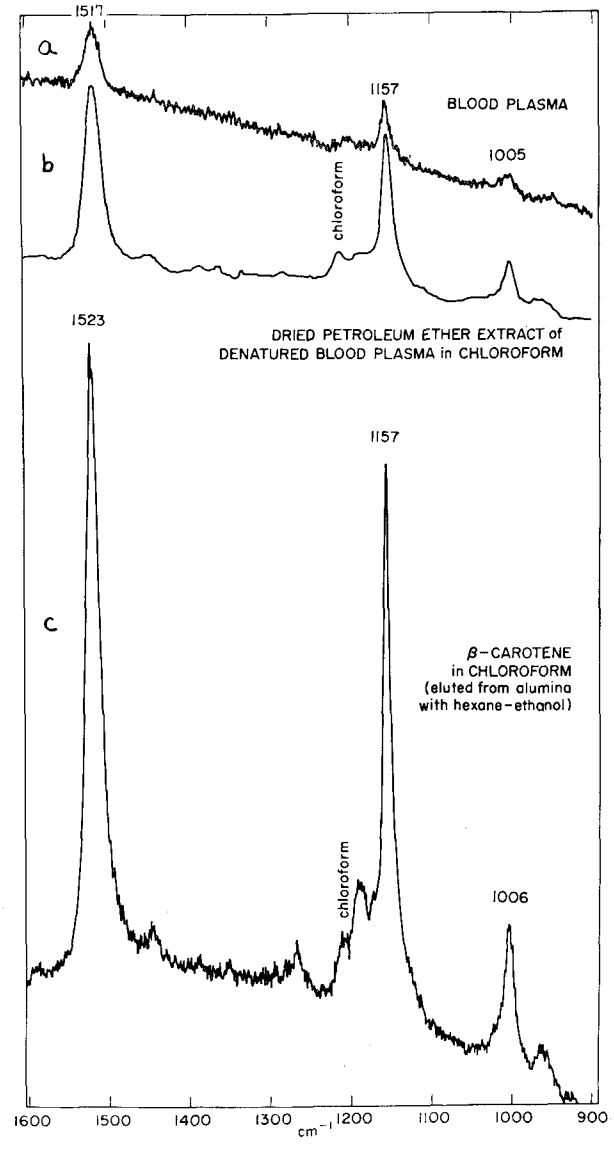
Resonance Raman spectra (cm^{-1}) in chloroform

	β-Carotene			Lycopene		Xanthophylls	
	Lit.[2,3]	Authentic sample[a]	Fraction I	Lit.[2,3]	Fraction II	Authentic sample[b]	Fraction III
ν_2	1527	1523	1523	1516	1516	1525	1525
ν_1	1158	1157	1157	1156	1155	1158	1157
ν_4	1006	1006	1006	1004	1004	1007	1005

Visible absorption spectra (nm) in *n*-Hexane

	β-Carotene			Lycopene		Xanthophylls		
	Lit.[6]	Authentic sample[a]	Fraction I	Lit.[6]	Fraction II	Lit.[7]	Authentic sample[b]	Fraction III
1.	482	474	474	506	497	480	471	468
2.	451	449	449	476	467	450	443	441
3.	430	427	427	447	438	–	421	421

[a] All *trans*-β-carotene (Aldrich); [b] Vegetable lutein (ICN).



Resonance Raman enhanced vibrational spectra of a) blood plasma (heparin, subjects fasted 12 h); b) petroleum ether extract of alcohol denatured blood plasma in chloroform, and c) β-carotene (Aldrich) in chloroform. Excitation by 5145 Å from argon ion laser, power 20–100 mW, scattered radiation analyzed at right angles to incident beam by Cary 82 triple monochromator. Typical spectrophotomer settings were: spectral band width 3 cm^{-1}, scan speed 2 cm^{-1}/sec and pen period 1 sec.

intensity ratios compare very favorably with those for *trans*-β-carotene which are $\nu_2 = 1523$, $\nu_1 = 1157$ and $\nu_4 = 1006$ cm^{-1} (Figure c), and are similar to the resonance Raman spectra of other carotenoid species[2,3].

Since carotenoid protein binding might influence the observed spectra, extraction and fractionation experiments were carried out[5]. Human blood plasma was denatured with methanol and the resultant suspension extracted with petroleum ether. The Raman spectrum of the concentrated petroleum phase (Figure b) showed the expected intensifications of the three carotenoid bands which occurred at frequencies identical to those of untreated blood plasma (Figure a).

Fractionation experiments were carried out to determine the contribution of specific carotenoids to the total Raman spectra. Three colored bands were obtained by a chromatographic method employing eluants of increasing polarity[5].

The resonance Raman and visible absorption spectra of the first fraction compared favorably to published spectra of β-carotene[2,3,6] and were found to be identical to an authentic sample recorded in this laboratory (Table and Figure c). The resonance Raman spectrum of the second fraction was identical to the published spectrum of lycopene, and its visible absorption spectrum compared favorably to literature values[2,3,6] (Table). Similarly, the third fraction was in agreement with the resonance Raman and visible absorption data for the xanthophylls[5–7] (Table).

As indicated above, the current method for the detection and quantification of carotenes involves a time consuming procedure to extract the carotenoids from blood plasma. The carotene level cannot be directly detected in blood plasma using visible absorption measurements because of interference by other colored components, e.g. bilirubin, which absorb in the same region. Thus, the Raman spectrum (Figure a) showing the small but distinct carotenoid bands of blood plasma is the first demonstration of direct detection by any method.

The original work of Larsson and Hellgren[1] indicated that the intensity of the bands as well as the fluorescence background in blood plasma change drastically

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in relation to the state of health of the individual. It is well known[8,9] that carotenoid and vitamin A levels in blood are directly related to diseases which affect the sites of carotene metabolism such as the small intestines and liver. It may become possible, therefore, to both detect and investigate very specific disease states by examination of blood plasma using the rather unusual technique of resonance Raman spectroscopy. Preliminary investigations of Rhesus monkey plasma in our laboratory gave spectra similar to the resonance Raman spectra of human blood plasma. That subject and the relationship of Raman spectra to further studies in human and animal health will be the topic of future communications. We thank Dr. D. CARLO, P. KNISKERN and J. JACKSON for providing the Rhesus monkey blood.

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Preparation of a Specific Ligand for the Purification of Arylsulfatases by Affinity Chromatography

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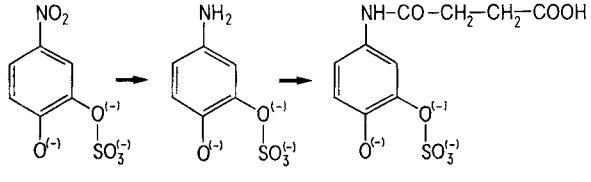
Summary. The reduction of *p*-nitrocatechol sulfate, the artificial substrate of arylsulfatases followed by a condensation of a succinic anhydride, gives a ligand for the purification of the enzyme by affinity chromatography.

To purify arylsulfatases (EC 3.1.6.1.), several methods were reported, using acetone precipitation[2], C.M. Sephadex, G200 Sephadex, etc. Unfortunately, they involved numerous steps, and active enzyme was generally obtained in poor yield.

Affinity chromatography seemed to be an attractive method to purify arylsulfatases from small quantities of tissues. It was first tried by SLOAN et al.[3] using psychosine sulphate as ligand, but during this step the enzyme was purified twice only. Moreover, psychosine sulphate is not readily available because the sulfate group is very sensitive to the acidic medium required for the cleavage of the fatty acid[4].

We have preferred to use the artificial substrate of the enzyme, *p*-nitrocatechol sulfate (I) (*p*-NCS). This compound cannot be used as a ligand as such because it has no amino or carboxyl group to bind to the Sepharose 4B CNBr activated matrix; the reduction of the nitro group allowed to reach the required amino group (*p*-aminocatechol sulfate: *p*-ACS). The well-known instability of such aminocatechols to oxydation led us to protect it by forming an amido group. This is the reason why we have condensed a succinyl chain on the *p*-ACS. The resultant amide should be much less oxidizable than the starting amine. The two steps of this synthesis are shown in the Formulae.



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The instability of the starting product limited the choice of the methods used for the reduction of the nitro group. In acidic medium, sulfate group is hydrolyzed[3], while in basic medium this aminophenol is quickly oxidized; any increase in temperature favours this oxidization. The insolubility of the starting material in most of the organic solvents excluded the reduction in anhydrous medium: the use of mixed metal hydrids seemed hazardous. Hydrogenation over 5% Pd/C at 50 °C and 50 bars in aqueous ethanol 50:50 (v:v) gives tars. Finally we used 98% hydrazine hydrate over Raney Nickel in aqueous solution[5], which lead to the amino compound. The condensation of the succinyl chain on the *p*-ACS prevented effectively the oxydation of the ligand which must be kept dry. Indeed, a slow darkening was observed after several days in aqueous solution, corresponding to an hydrolysis of the sulfate group (as seen by IR).

Experimental. General procedure. *p*-nitrocatechol sulfate dipotassium salt was supplied from Sigma Co. UV-spectra (water) were recorded on a Varian 635 M Spectrophotometer. IR-spectra were performed as KBr pellets using a Perkin Elmer 457 Spectrophotometer and NMR-spectra on a Jeol C 60 HL apparatus using T.M.S. as an internal standard.

p-Aminocatechol sulfate. 98% hydrazine hydrate (0.9 ml) and Raney Nickel (Merck) (1 g) were added to an aqueous solution (20 ml) of *p*-nitrocatechol sulfate (500 mg). The mixture was stirred during 5 min until complete decolouration. After filtration, lyophilization gave a grey residue (II). UV (water): 245 nm; IR: 3380–3300 (NH), 1600 (aromatic C=C), 1245 and 1050 (sulfate group), 810 (aromatic C–H) cm^{-1}.

p-Succinylaminocatechol sulfate. *N*-ethyl, *N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (400 mg) and succinyl anhydride (300 mg) were added to an aqueous solution (20 ml) of the preceding crude *p*-aminocatechol sulfate. The mixture was stirred for 2 h at room temperature and lyophilized. The residue was washed with diethylether (4 × 20 ml) and dried with a stream of nitrogen. This compound (III) presented the following absorptions: UV (H_2O) λ_{max}: 235 nm. IR: 3500 (OH), 3480 (NH), 1725 (acid), 1675 (amid), 1245 and 1050 (sulfate group), 810 (aromatic C–H) cm^{-1}. NMR (D.M.S.O.d6): δ2,1 (m,4H CH2),6,5(m,3H aromatic H).

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