Table III. Solubility measurements

Suspension identification	Solid: solution ratio g/l	pH	Solubility (ppm solution) Ca	P	Ca:P mole ratio of dissolution	pK sp of hydroxy-apatite[a]
LRS	0.36	7.34	4.05	1.46	2.10	111.0
LRS	0.71	7.39	4.55	1.39	2.58	110.1
NBS	0.36	7.15	2.65	1.06	1.94	115.6
NBS	0.71	7.14	3.00	1.26	1.85	114.8
LRS + H_3PO_4	1.0	6.55	8.10	7.95		113.0
LRS + H_3PO_4	1.0	6.26	13.54	16.66		112.8
NBS + H_3PO_4	1.0	6.23	7.66	9.47		117.0
NBS + H_3PO_4	1.0	5.88	13.10	18.04		117.9

[a] Calculated according to CHAVERRI and BLACK[11].

greater Ca:P ratios in the solution than in the solid suggest that the excess calcium in the solid was greatest on or near the surface of the hydroxyapatite crystals[11].

Isoionic exchange. The results of the isoionic exchange determinations are given in Figure 3 in which the logarithm of percentage ^{32}P remaining in solution is plotted as a function of the logarithm of time.

In the case of NBS hydroxyapatite, only one linear relationship between log ^{32}P in solution and log time was observed during the experimental period of 90 h. The negative slope of this relationship was 0.027 and 0.019 respectively for 0.5 and 0.25 g hydroxyapatite per 700 ml of solution. In the case of the LRS hydroxyapatite, two linear relationships were observed in the same period. The negative slopes of these were 0.127 (I), 0.076 (II) and 0.108 (I), 0.053 (II) respectively for 0.5 and 0.25 g hydroxyapatite per 700 ml solution. Thus, only one reaction mechanism seems to be involved in the exchange of phosphate ions between the NBS hydroxyapatite and the bathing solution while two reaction mechanisms appear to be operating in the case of the LRS hydroxyapatite.

'Surface-exchangeable P' calculated according to OLSEN[6] was 8.25 and 8.40 mg P/g respectively for 0.5 and 0.25 g/700 ml suspensions of LRS hydroxyapatite and 0.80 and 0.83 mg P/g for similar suspensions of the NBS hydroxyapatite. The LRS hydroxyapatite appeared to have about 10 times more 'surface-exchangeable P' than the NBS hydroxyapatite, but the solid/solution ratio had little or no effect for either material.

OLSEN's conversion factor of 4.20 was used to transform surface-exchangeable P to specific surface area (Table II). Good agreement between the specific surface area measured by the continuous flow technique and the ^{32}P method was obtained for the LRS hydroxyapatite but not for the NBS material, suggesting that the factor differs between preparations.

Measurements of the surface area of finely divided solids are, however, notoriously unreliable and too much significance should not be attached to them. The relative surface areas, i.e. the specific surface area of the LRS hydroxyapatite divided by the specific surface area of the NBS hydroxyapatite could be more informative. This ratio is 2.7, 3.4 and 10.2 when determined respectively from the continuous gas flow technique, electron micrographs and the ^{32}P exchange method. The much higher ratio obtained by the last method suggests that the isotopic exchange process differed between the 2 preparations. It is probable that much more crystal growth would occur in the suspension of the less perfect LRS crystals than in the suspension of the near-perfect NBS crystals. Thus in the case of the LRS hydroxyapatite, loss of ^{32}P is likely to result from recrystallization as well as from surface exchange while in the NBS preparation only the second mechanism would be important.

Zusammenfassung. Es wurden sowohl ältere als auch neuere Verfahren zur Fällung von Kalziumhydroxyapatit aus wässriger Lösung vergleichend überprüft und die erhaltenen Präparate mittels chemischer und physikalischer Methoden charakterisiert, wobei sich ergab, dass das vom National Bureau of Standards entwickelte Verfahren die am besten definierten Kristalle liefert.

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Fisons Limited – Fertilizer Division, Levington Research Station, Ipswich, Suffolk (England), 15 October 1970.

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PRO EXPERIMENTIS

Enzymatic Sulfation of 4-Methylumbelliferone

Enzymatic sulfation of aromatic hydroxy compounds is mostly investigated by using *p*-nitrophenol as the sulfate acceptor. This method, described by ROY[1], makes use of the solubility of the methyleneblue complex of the sulfuric acid ester. We developed a method for the demonstration of sulfating activity using one of the most fluorescent molecules known, 4-methylumbelliferone, 4-methyl-7-hydroxy-coumarin (MU). Various derivatives of MU are in use for the assay of hydrolases, but, so far as we know, no attempt has been made to use the parent

substance for demonstration of the enzymatic formation of the sulfate derivative.

Materials and methods. 4-Methylumbelliferone sulfate (MUS) was synthesized by the method of SHERMAN and STANFIELD[2], or bought (Koch-Light). No difference between the two products was observed.

From ATP and sulfate-ions in the presence of Mg^{2+}, the enzymatic formation of active sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is possible. Such a PAPS-generating system was prepared in the lyophilized form from sheep brain[3]. For the activation and for the transfer of sulfate from PAPS to the accepting phenol, we used a lyophilized preparation from rat liver[4]. This preparation contains, however, both the sulfate activating and transferring enzymes. The former can be inhibited by the addition of EDTA to the incubation mixture. In this way it is possible to use this preparation for both reactions.

Our incusation circumstances do not differ essentially from those used by others for the sulfation of other compounds e.g.[3-6] and the conditions are optimal for the system described here. The sulfate activating enzyme system in our experiments contained 21 mg enzyme preparation, either from sheep brain or from rat liver, in 4.5 ml *tris*-HCl buffer 0.4 *M*, pH 7.4, containing 2.2 m*M* $MgCl_2$, 11 m*M* K_2SO_4 and 3.3 m*M* ATP. After incubation for 1 h at 37 °C the raction was stopped by heating for 60 sec in boiling water. After cooling and centrifugation 1.5 ml of the clear supernatant was added to 1 ml of a solution of MU and EDTA in *tris*-HCl buffer 0.4 *M*, pH 7.4, containing 14 mg rat liver preparation.

The final concentration of MU being 0.4 m*M* and of EDTA 50 m*M*. This mixture was incubated at 37 °C for 45 min. The reaction was stopped by heating in a boiling water-bath for 1 min. The supernatant was collected after centrifugation. This fluid was investigated for the presence of MUS.

Results. Thin-layer chromatography on silicagel (Merck, Darmstadt, 1.5 mm thickness) revealed under UV-light one spot, MU. To detect MUS on the chromatogram the thin-layer plates were suspended in fumes of HCl in a closed vessel for approximately 10 min. The chromatograms were then exposed to a current of air for a short period, thereafter they were examined under UV-light again. In this way MUS was hydrolyzed and could be detected as MU.

In the Table the Rf values of authentic and enzymatically formed MUS are presented, after thin-layer chromatography in some developing fluids. As can be seen from this Table, the Rf values of authentic and enzymatically formed MUS are in excellent agreement.

After electrophoresis on cellulose acetate (Gelman, Sepraphore III) at pH 8.6 (veronal buffer 0.05 *M*) at 350 V for 10 min, MU was left at the application line, MUS being displaced some centimeters to the anode. Hydrolysis by HCl fumes revealed MUS as MU, being visible under UV-light. The product formed by incubation appeared at the same place as authentic MUS.

Hydrolysis of the formed product with a highly specific arylsulfatase (Sigma, type III) revealed again MU, as demonstrated by chromatography and electrophoresis. Next to all this, the formed MUS and the hydrolysis products exhibit the same excitation and emission spectra on an Aminco-Bowman spectrofluorometer as do the authentic compounds.

Discussion. From all these experiments we consider the enzymatic formation of MUS proved. In other experiments we investigated the essentiality of the cofactors mentioned above. ATP, SO_4^{2-} and Mg^{2+} were found to be necessary for the activity of the system, as were, of course, the enzyme preparations. We reached the conclusion, based on the above-mentioned results, that the development of a very sensitive assay of sulfotransferase (E.C. 2.8.2) should be possible. The only question to be solved remains the quantitation of the amount of MUS formed. Preliminary experiments in our laboratory promise excellent results from the use of a Dowex H^+ cation exchanger retaining MU, all of the MUS passing the column. After acid hydrolysis the formed MUS can be very sensitively measured as MU[8].

Zusammenfassung. Die Aktivität von Sulfo-Transferasen wird mit Hilfe der 4-Methylumbelliferon-Methode nachgewiesen.

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TLC of authentic and enzymatically formed MUS and of MU

Developing fluid	Rf value MUS Formed	MUS Added	MU
Butanol-ethanol-ethylmethylketon-water (3:3:3:1)[7]	0.6	0.6	0.8
Butanol-ethanol-ethylmethylketon water (4:2:3:1)	0.3	0.3	0.8
Butanol-ethanol-ethylmethylketon (4:2:3) saturated with water	0.4	0.4	0.8
Butanol-ethanol-water (3:3:1)	0.8	0.8	1.0
Butanol-acetic acid-water (10:2:5)	0.6	0.7	1.0

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A Method for Determining the Maximal Stimulus Strength for Massive Stimulation of Skeletal Muscle

Massive stimulation with square pulses is now widely used in experiments where the simultaneous excitation of all parts of a muscle, or a muscle fiber preparation, is desired[1-4]. The preparation is usually placed longitudinally between 2 parallel platinum or silver-silver chloride plate electrodes surrounded by a physiological salt solution. Excitation is effected by passing brief electric pulses through the solution.

The suitability of a stimulator for maximal massive stimulation is usually tested by determining if the