	Cases	Basal	1/2 hour	1 hour	2 hours	Amounts in
Triglycerides	9	145 ± 50.48	179.55 ± 76.46	177.66 ± 55.14	147.22 ± 55.27	mg/100 ml
Free fatty acids	9	0.42 ± 0.15	0.30 ± 0.16	0.26 ± 0.17	0.33 ± 0.17	mEq/l
Total cholesterol	8–9	166.00 ± 28.96	188.88 ± 49.49	183.50 ± 48.99	178.50 ± 57.82	mg/100 ml
Blood sugar	9	89.47 ± 14.8	100.57 ± 12.82	91.57 ± 12.73	85.70 ± 15.42	mg/100 ml
Insuline	9	30.11 ± 13.27	49.88 ± 14.32	47.66 ± 16.85	32.44 ± 9.11	μU/ml

were taken 1/2, 1 and 2 h later. The following were estimated in each sample: free fatty acids[11–13], total cholesterol[14,15], triglycerides[16], blood sugar[17,18] and serum insulin[19]. The total cholesterol in the basal sample and in the 1-h and 2-h samples could only be checked in 8 patients. For statistical treatment, the results were analyzed by Student's *t*-test.

Comments. The results are shown in the Table. There were no significant variations in serum-cholesterol, serum-triglycerides and glucose concentrations. The free fatty acids decreased significantly after 1 h (p 0.05). Serum insulin increased significantly after 1/2 h ($p < 0.01$) and continued to be high after 1 h ($p < 0.05$). After 2 h the basal concentrations were restored.

From the foregoing it may be deduced that galactose does not exert any influence on the serum cholesterol, triglycerides or glucose concentrations. It does, however, provide an effective stimulus for the secretion of insulin, resulting in an appreciable antilipolytic effect as demonstrated by the decrease of free fatty acids, a fact which points to the possible existence of pancreatic receptors for galactose or to the production by the gut of some substance which increases the blood sugar content when galactose is present.

Resumen. La administracion de 100 g de galactosa a 9 voluntarios sanos por via oral, demostro ser capaz de elevar la insulinemia, sin modificar la glucosa en sangre. Secundariamente se aprecio un descenso de los acidos grasos libres. La galactosa puede actuar directamente sobre el pancreas o a traves del intestino.

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Calcium in Myonuclei: Electron Microprobe X-Ray Analysis

Our previous work with electron microprobe X-ray analysis of embedded thin sections of skeletal muscle and myocardium has shown that calcium can be detected and localized at a subcellular level and that, indeed, the highest intracellular concentration of the element is in the nucleus[1,2]. Here we report further studies of the calcium distribution, in particular within the nucleus, and studies of the changes occurring in conditions known to increase intracellular calcium[3]. We have been especially concerned in this recent work to see how the observed distributions are affected by different methods of specimen preparation.

Three types of preparation were studied. a) Frogs' sartorius muscles were fixed and embedded in various ways (some are shown in Table I). b) Hearts of rats, either untreated (controls) or injected with isoproteranol (100 mg/kg i.p. 6 h before sacrifice), were fixed in a mixture of 1% osmium and 2% pyroantimonate ($K_2H_2Sb_2 \cdot 4H_2O$)[3]. The embedding was either conventional in Epon 812 or in a polymerizable mixture of 50% glutaraldehyde and urea in which dehydration is omitted[4]. c) Biopsies were taken from the heart of an open-chested dog during slow and fast heart rates induced by electrical pacing[5]. The tissue was fixed in a mixture of osmium plus pyroantimonate, as in b), dehydrated, and embedded in Epon 812.

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[5] The open chest dogs' hearts experiments were done in the Hadassah laboratory of cardiac physiology as part of a study on myocardial calcium fluxes now in preparation with the collaboration of Dr. J. KEDEM and P. S. ROGEL.

Table 1. Calcium analyses of frog muscle fixed in various ways

Fixation	Embedding	Area	$[P\text{-}B]/B$ in 100 sec (energy dispersive analysis)	C_{Ca} in %[b] (crystal diffractive analysis)
$^1/_2$ h in 2% glutaraldehyde +0.2 *M* cacodylate buffer	Glutaraldehyde + urea	Nucleus dark Nucleus pale Cytoplasm	0.31 (2.3)[c] 0.13 none detected	0.48 (1.8)[c] 0.27
1 h in 1% OsO_4 + 1% oxalate + 0.1 *M* cacodylate buffer	Glutaraldehyde + urea	Nucleus dark Nucleus pale Cytoplasm Cell wall	0.92 (1.4) 0.66 0.47 0.52	not done
Osmium + pyroantimonate solution	Glutaraldehyde + urea	Nucleus dark Nucleus pale Cytoplasm	< 0.49[a] (2.3) < 0.21 < 0.28	0.54 (2.4) 0.23
Osmium + pyroantimonate solution	dehydration and epon	Nucleus dark Nucleus pale Cytoplasm	< 0.25 (3.1) < 0.08 < 0.14	0.41 (1.8) 0.23

[a] Ca concentration is less than tabulated here since the tabulated figure for energy-dispersive analyses includes both Ca and Sb radiation in the case of material fixed in pyroantimonate. [b] C_{Ca} is the local mass fraction, i.e. g of calcium per g of tissue, in the form in which the specimen exists during analysis. Beam-induced loss of organic mass may raise the values above the true mass fractions prior to analysis. [c] Figures in brackets give the ratios of Ca readings in dark and pale areas of the nucleus. Energy-disperse and diffracting-crystal measurements on different fields.

Thin sections (100–150 nm) were cut from all the samples on to formvar and carbon-coated grids. They were examined unstained with the EMMA-4 analytical electron microscope (AEI Scientific Apparatus, Ltd., England). The principles of X-ray microanalysis are now generally well known and many reports of work with the EMMA-4 exist[6,7].

For the detection of calcium X-rays, both a diffracting crystal spectrometer with penta-erythritol diffractor and a Kevex Si(Li) 'energy-dispersive' spectrometer were employed. The diffracting system selects radiation according to wave length with excellent discrimination (fully rejecting antimony radiation, for example, when set for calcium). The energy-dispersive system, which is based on the conversion of X-ray quantum energies to electrical pulse heights, is not so discriminating but does record simultaneously most of the X-ray spectrum. An accelerating voltage of 40 kV was used, with probe currents between 10 and 80 nA and a probe diameter of approximately 200 nm. 20-sec recordings were made of peak count (P) with the diffracting crystal spectrometer set for calcium K_α, and background (B) offset from the calcium position. The 'white' radiation (W) was noted and corrections were made for the supporting film (W_b). The data allowed the calculation of relative mass fractions ($R = [P-B]/[W-Wb]$) and the calcium concentration in each microvolume analyzed[8]. Spectra displayed on the TV display of the Si(Li) detector were photographed after 100-sec analysis. The calcium K_α X-rays were analyzed statistically by subtraction of the background from peak integrals, and the value $[P-B]/B$ was calculated as an alternative measure of relative calcium mass fraction where continuum counts W were not separately recorded. The figures given in the Tables are averages of 2–4 readings with a moderate scatter which is partly due to technical difficulties of beam localization[2]. Thus the results are

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Table II. Relative mass fractions of calcium in Os + Sb fixed myocardium

Specimen	Embedding	R Specimen normalized to R Standard	
		Nucleus	Sarcomere
Rat heart control	Dehydration and epon	0.0148	0.0012
Rat heart control	Glutaraldehyde + urea	0.0269	0.0115
Rat heart 6 h after isoproteranol	Dehydration and epon	0.0141	0.0045
Rat heart 6 h after isoproteranol	Glutaraldehyde + urea	0.0160	0.0450
Dogs heart slow rate	Dehydration and epon	[a](17) 0.0306 ± 0.0116	(19) 0.0056 ± 0.0059
Dogs heart fast rate	Dehydration and epon	[a](14) 0.0213 ± 0.0063	(21) 0.0127 ± 0.0054

[a] in brackets – number of areas analyzed; in rats hearts the figure is an average of 2–4 areas,

only semi-quantitative. Nevertheless, they seem to be within the range of calcium values obtained by other methods[9–13].

Irrespective of the method of tissue preparation, calcium is easily detected in myonuclei (Table I). Extranuclear calcium could be measured with EMMA-4 only after osmium fixation. Glutaraldehyde fixation or pyroantimonate alone[1], at a pH of 7–7.6, do not lead to cytoplasmic detection. Embedding in glutaraldehyde and urea gives higher calcium X-ray emission than the same specimen treated conventionally (Table II). It appears that alcohol dehydration produces a calcium efflux even after the cation has been precipitated with pyroantimonate.

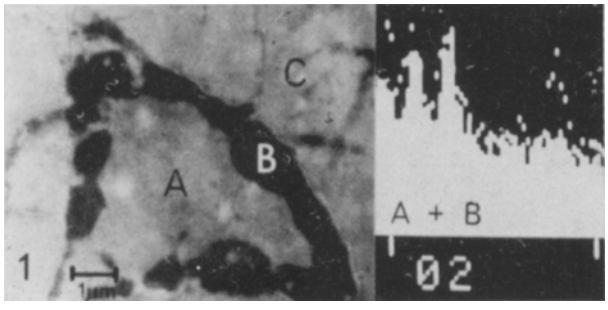
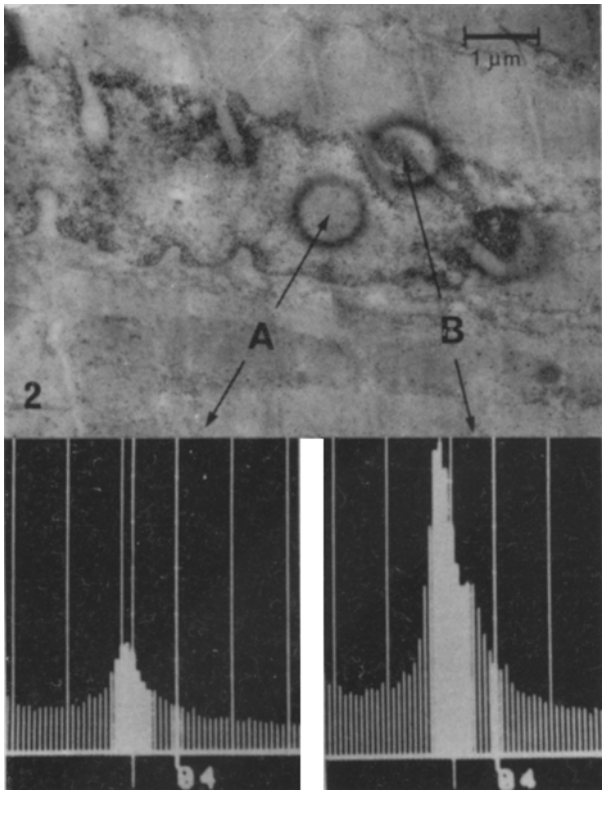


Fig. 1. Transverse section of frog's sartorius. Minimal preparation, glutaraldehyde and urea embedding, unstained 150 nm section. The nucleus has pale euchromatin (A) and dark heterochromatin (B). The sarcoplasm (C) has poor contrast. On right: Photo of superposed X-ray spectra in the 2–4 keV range, obtained with 100 sec analysis, using the Si(Li) detector. The spectrum from the pale area A is displayed in solid white, while the spectrum from the dark area B is displayed by the dots. The 4 readily observed peaks, going from left to right, are sulphur at 2.31 keV, chlorine at 2.62 keV, potassium at 3.31 keV and calcium at 3.69 keV. The calcium peak stands out in the dotted spectrum from the heterochromatin but is only marginally detectable in the other spectrum.



What also emerges in Table I is that electron-dense parts of the nucleus (heterochromatin of the margins and the nucleolus) have a much higher concentration of calcium than other locations. The ratio between calcium emissions from the dark and pale parts of the nucleus varies with specimen preparation, being lowest after osmium and oxalate fixation. Specimens which were treated minimally (half an hour's fixation in 2% glutaraldehyde with 0.2 *M* cacodylate buffer, followed directly by embedding in 50% glutaraldehyde and urea) had very little contrast (Figure 1). Nevertheless, the nuclei could be visualized and the different calcium localizations could be demonstrated. The preponderance of calcium in the heterochromatin as compared with euchromatin was also seen in myocardial nuclei (Figure 2).

The information obtained from the various experiments on myocardia of rats and dogs (all fixed in the osmium and pyroantimonate mixture) is summarized in Table II. In the nucleus only the readings from regions rich in heterochromatin are recorded. Once again, more calcium is retained in tissues not subjected to alcohol dehydration.

The results given as relative calcium mass fractions indicate that 6 h after an i.p. injection of isoproteranol there is an enormous increase in myofibrillar calcium, while the nuclear calcium decreases.

Similar increased calcium concentration in sarcomeres and decreased myonuclear concentration were found when the dog's heart rate was altered from very slow (70 beats per min) to very fast (250 beats per min). The cell nucleus has a significant concentration of inorganic salts, thought to be related to ribonucleoprotein particles and to RNA synthesis[14]. The calcium concentration in the nucleus appears to be at least twice that elsewhere in the cell[10,11], and isolated myonuclei can take up and bind calcium from bathing solutions against a steep chemical gradient[11]. Although the specific function of myonuclear calcium is unknown, a role in energy inactivating processes and amino acid transport has been postulated[11].

These decreases in myonuclear calcium, in conditions where intracellular calcium increases, are difficult to interpret. It may only mean that there was a dispersal of the cation in the nucleoplasm, leading to lower counts;

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Fig. 2. Dog's myocardium fixed with osmium and pyroantimonate. Examination without a decontaminator left burn marks at the sites of analysis. The nucleus has pale, chromatin-poor 'euchromatin' areas (A) and dense chromatin-rich 'heterochromatin' areas (B). The illuminated area in each spectrum is centred on the calcium $K\alpha$ line at 3.69 keV, as can be seen from the position of the centre-line of the band, but the spectral peaks are shifted to the left by the contribution of the antimony α line at 3.60 keV. There is no problem in resolving these elements with a diffracting crystal spectrometer.

then again, it may be that the intranuclear calcium takes part in intracellular calcium kinetics. Whether the changes are related to mechanical muscle activity or are an expression of adaptation processes remains unclear.

[15] We acknowledge the technical assistance of M. SCRIPPS and S. SCHWARTZ. We thank B. GOLEK for editorial help. This research was supported by the British Science Research Council, the Joint Research Fund of the Hebrew University-Hadassah Medical School, and an EMBO travelling fellowship for R.Y.

Résumé. L'analyse par microsonde électronique des cellules musculaires montre de hautes concentrations de calcium dans les noyaux; la concentration peut être modifiée pharmacologiquement ou par «pacemaker» électrique

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Organophosphate-Detoxicating Enzymes in *E. coli*. Gelfiltration and Isoelectric Focusing of DFPase, Paraoxonase and Unspecific Phosphohydrolases

Toxic organophosphates, commonly known as insecticides or as nerve gases, are inhibitors of cholinesterases, thereby blocking nerve transmission[1]. Some hydrolytic enzymes ('phosphorylphosphatases') are capable of splitting the acidanhydride bond of organophosphate poisons, forming non-toxic organophosphorus acid[2,3]. The following paper describes the enzymatic organophosphate detoxication by *E. coli* phosphohydrolases. Two detoxicating enzymes were found, a DFPase (E.C. 3.8.2.1) and a paraoxonase (arylesterase, E.C. 3.1.1.2).

DFPase-activity was determined by 3 methods with di-isopropylfluorophosphate (DFP) as substrate: titrimetrically[4], manometrically[5] or by measuring the liberated fluoride with an ionsensitive electrode[6]. Paraoxonase was tested colorimetrically with 0,0-diethyl-0-*p*-nitrophenylphosphate (paraoxon)[7]. Alkaline and acid phosphatase were measured colorimetrically with *p*-nitrophenylphosphate in 0.2 *M Tris*-buffer at pH 9 and pH 6, the absorbance at 405 nm was read after diluting samples with 1 *M* sodium hydroxide. Phosphodiesterase determinations were performed with *bis-p*-nitrophenylphosphate at pH 8 in the same way. All tests were performed at 25 °C, activity is expressed as 1 μmole substrate hydrolyzed per min. *E. coli* K_{12}sr extracts were prepared according to MCILWAIN[8]. The extract was fractionated by gelfiltration on a Sephadex G-200 column, the elution diagram for protein und DFPase is shown in Figure 1. The distribution coefficients (K_d-valus) of the 2 DFPase peaks were calculated[9] and compared with those of other phosphohydrolases in a second experiment on the same column (Table I).

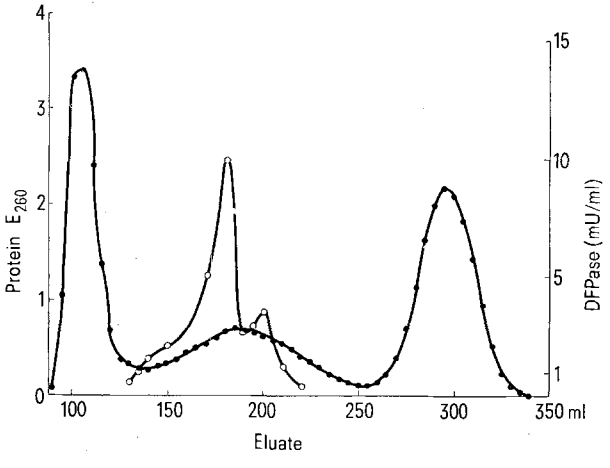

Fig. 1. Gelfiltration of *E. coli* extract on Sephadex G-200 (2.5 × 73 cm). 4 ml extract (2 g wet cells) containing 73 mg protein and 562 mU DFPase were fractionated at 1°C, flow rate: 0.2 ml/min. ●, protein ($E^{1\,cm}_{260}$); ○, DFPase (mU/ml).

DFPase and paraoxonase could clearly be seperated by gelfiltration; no overlapping activity was detected: DFPase fractions did not hydrolyze paraoxon and vice versa. There was no hydrolysis of *p*-nitrophenylphosphate at acid or alkaline pH or of *bis-p*-nitro-phenylphosphate by either the DFPase or paraoxonase containing fractions. The phosphohydrolases in *E. coli* extracts were additionally subjected to free preparative isoelectric focusing[10,11]. Four DFPase peaks with isoelectric points of 5.3, 5.7, 6.1 and 7.8 were found (Figure 2). The isoelectric points of the other phosphohydrolases were determined in an identical way in a different experiment, the isoelectric points are listed in Table II. The phosphohydrolases of *E. coli* showed similar or even identical isoelectric points, so, in contrast to the gelfiltration experiment, they could not be separated by this method.

Coli DFPase has a low substrate affinity (Km 1.7×10^{-2} *M*/l for DFP) and shows pure MICHAELIS-MENTEN kinetic (Figure 3). The pH-dependence of *coli* DFPase is shown

Table I. Distribution coefficients (K_d-valus) of *E. coli* phosphohydrolases on Sephadex G-200 (2.5 × 73 cm)

Enzyme	K_d-valus		
DFPase	0.21	0.31	
Paraoxonase	0.11	0.55	0.66
Acid phosphatase	0.39		
Alkaline phosphatase	0.42		
Phosphodiesterase	0.40		

Table II. Isoelectric points of *E. coli* phosphohydrolases determined by free preparative isoelectric focusing in 2% ampholine, pH 3–6

Enzyme	Isoelectric points			
DFPase	5.3	5.7	6.1	7.8
Paraoxonase	5.3	5.6	6.2	
Acid phosphatase	4.9	5.3	6.4	
Alkaline phosphatase	4.8	5.3		
Phosphodiesterase	5.0	5.3	5.6	6.3