Instability of the terminal bond of ATP, phosphocreatine and arginine phosphate

by Harold Hillman

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Abstract

Adenosine triphosphate; (ATP) and phosphocreatine (PCr) both at 37°C, and arginine phosphate, (PA) at 22°C, in 2 mM solutions at pH 7.3-7.5, absorbed visible light slightly, and released inorganic phosphate non-linearly. The quantity of inorganic phosphate extractable depended non-linearly, and on the time since subjection to, the following agents: light (500 lux; 2-20 sec; 100-1000 lux; wave-length, 313-578 mμ); sound (256 c/s, 100-1000 mW into a loudspeaker 1 metre away, fixed power, 100-1000 c/s); electromagnetic field around the test tubes 50 c/s **(** 48 watts, 2-20 sec; 24-240V); centrifugation (200 g 1 min, 1000 rev/min.). The extractable free phosphate was also dependent on the Na^{+}, not $K,^{+}$ concentration.

Introduction

During the course of experiments examining the ability of the retina to break down ATP, it was noticed that, at 37^{0}C, the ATP itself was sensitive to visible light. It was further found that ATP was similarly sensitive to sound, electrical energy, centrifugation and Na^{+} concentration. PCr at 37°C also re-acted to the same agents. Since the phosphate of high transfer potential in poikilotherms is PA (Lohmann, 1935), it was thought possible that this substance might exhibit these same properties at room temperature (22°C*).* This proved to be the case.

Methods

$Na_2ATP,3H_20$ (Sigma), phosphocreatine $4\ H_20$ (Sigma), or Na arginine phosphate, were dissolved in water to approximately 4 mM, neutralized to pH 7.3- 7.5 by addition of about a third of the total volume of 0.6 M tris buffer. The PA was a very pure sample kindly provided by Dr. J. Morrison of the Australian National University, through the good offices of Prof. A. Ennor. It had been prepared by the method of Ennor, Morrison and Rosenberg (1956). It was diluted to make a 2 mM solution, which had a pH of 7.3-7.5. Aliquots, 0.5 ml of the 20 ml made up, were pipetted into test tubes. Usually 24 such tubes containing ATP or PCr were taken to a thermostatically controlled room at a temperature between 36°C and 37°C, in the dark. The 24 tubes were kept in the quiet for at least 20 min, before the experiments were carried out. This allowed them and the solutions to warm to 37°C. The PA was placed under a black cloth in a quiet laboratory at a temperature of 22°-23°C. Meanwhile, to each of 24 test tubes, was added a mixture of, 2.0 ml of 1 N H_2S0_4, 0.5 ml of 5% ammonium molybdate, and 3 ml of isobutanol, and they were placed in ice. At the beginning of the experiment, 2 or 4 controls were taken, and then the light, sound or other agent, applied. As soon as possible after that, the 5.5 mls of cold contents of the tubes were decanted into the test tubes in the dark, and shaken vigorously 2-3 times. The test tubes were then put on ice in a large glass container in a black box. The addition of the reagents to the phosphate solution took about 1 sec, and placing them on ice took less than 5 sec. Each extraction under a particular condition was replicated by two technicians at the same time, as can be seen from the graphs. As soon as possible after the end of the whole experiment - which took 5-20 minutes - the container with the stoppered tubes in ice was taken into a

laboratory at 20-23°C and in the least light in which it could be done (about 10 lux) the acid layer containing the high-energy phosphate, was pipetted out, and discarded. The quantity of inorganic phosphate in the isobutanol layer was then measured using a stannous chloride reagent by the method of Berenblum and Chain (1938). It was read at 700 mμ on a Zeiss (PM Q11) or a Beckman (G 2400) spectrophotometer, both of which had their own calibration curves. Linear calibration curves were made by taking 5- 800 μl of 0.1 mM Na_2HPO_4 with micropipettes, and extracting and measuring these quantities by the same procedure. All glassware was washed with 95- 98% H_2S0_4.Two to four experiments, each with simultaneous duplicate specimens throughout, were done, except for PA, where the shortage of material permitted sometimes only one double experiment, and the absorption of ATP on exposure to light, which was done 6 times (Fig. 3). The test tubes in which each of the reactions occurred were coded and analysed at random. The code was not revealed until after the quantities of phosphate released had been calculated.

Light

Light was obtained either from the commercial laboratory bench lamps which were 250 V, 60 W, Tungsram, of 726 lumen, or from an Osram mercury high pressure lamp, model HD, filtered through water and used with a Zeiss monochromator M4QII. The illumination from the former light source was measured in luxes using an AVO Light Meter, Model 2. The energy in watts coming from the latter source was kindly measured by Engineer B. Olander, of Chalmers Technical High School, using a Philips 90 CV photocell, calibrated by the makers. Throughout this paper, the commercial lamp source will be referred to as 'laboratory' lamp, and the mercury lamp with the monochromator giving light of known wave lengths, as 'mercury' lamp. The intensities of illumination due to the laboratory lamp were 0-1000 lux, chosen to cover the range 20-50 lux – the intensity recommended for - corridors up to 1000 lux recommended for operating theatres, or for minute detailed work.

Sound

The sound source was a Hewlett-Packard test oscillator model 650A, with a Telefunken audio amplifier, whose input went into a broad band loudspeaker (Sims PMB 8003). The linearity between the input and the output of the loudspeaker was tested using a Shure dynamic microphone, Model 55S, and a Tektronix 502 oscilloscope. Where a fixed frequency was used, it was 256 c/s. When the frequency was altered from 0-1000 c/s the energy output of the loudspeaker was kept constant at 50 mW, equal to that coming from the loudspeaker at 256 c/s, with 50 mW input. This was tested by recording the amplitude of the a.c. signal coming out of the crystal microphone on an oscilloscope as a result of the sound directed to it. When the intensity of the sound was measured, the 24 test tubes containing the phosphate were all put 1 metre away from the loudspeaker, and it was turned to zero input. The control phosphates were then extracted, and the input to the loudspeaker turned to 100 mW for 4 sec, at the end of which the phosphate of 2 further tubes was extracted, and the loudspeaker then quickly turned to zero. The input was then put at 200 mW, and after 4 sec, a further 2 tubes extracted, and so on. This procedure was adopted, as it was found impossible to screen completely those samples from the sound, which were not being extracted under that condition.

Electrical energy

A commercial loudspeaker with a resistance of 2500 Ω was found which had a hole of diameter 19 mm, in which a test tube stood vertically. Current was taken from the 240 V a.c. 50 c/s mains source to a Berco Rotary Regavolt variable resistor, type 42 A, and into the

coil. The input voltage to the coil could thus be controlled from 0 to 100 %. The alternating current thus induced an electromagnetic field in the 0.5 ml of solution in the test tube without the use of an electrode.

Centrifugation

In the dark at 37°C, 4 tubes were taken as controls, then 16 others placed in the Silverstolpe BEG 1100 centrifuge, and it was turned up to 1000 rev/min, so that, it was revolving for 1 min, including the time of turning it up go its maximum speed. It was then switched off and took between 50 and 55 sec to stop. At 60 sec after switching off, the first 2 samples were taken out, and the phosphate extracted, and they were placed in the cold. Two further samples were taken out every 30 sec.

The effect of Na^+ and K^+ concentrations

Stock solutions were made up containing concentrations of Na^+ or K^+ solutions between 0 and 150 mM.In the experiments where the effect of the ions was studied *separately*, the Na^+ or K^+ solutions were made up to 150 mM with sucrose. When the effect of the two ions was studied together, the total concentration of K^+ plus Na^+ was always 150 mM. Thus the solutions contained 150 mM concentration of sucrose, of Na^+,of $K,^+$ or of Na^+ plus K^+. The sample tubes were left in the dark at 37°C for 30 min, and then the phosphate was extracted and measured.

General technique of experiments

With light, sound and electrical currents, a linearly increasing quantity of energy was tested. They were also examined for exposure to these agents from 0 to 20 sec, at 2 sec intervals. The agent was always switched off and the phosphate reagents added as rapidly as possible. The tubes were then placed in the ice, in the dark room at 37°C. In the case of light, electric currents and centrifugation, the organic phosphates were subjected to these agents for 2-60 seconds, and examined at 37^0C for 3-5 min at 30 sec intervals thereafter.

Two sorts of controls were used: (i) without any physical or chemical reagents being applied to them, the 'high-energy' phosphates were extracted every 10 sec for 100 sec, and also every 1 min for 10 min to see if the extractability of the inorganic phosphate had a phasic component (Figs 1, 2); (ii) 0.1mM of Na_2HPO_4 was subjected to the same agents under the same conditions, and the phosphate extracted and measured in the same way. This was to see if the extraction process was itself affected significantly by the agents.

The absorption of similar 2 mM solutions of the organic and inorganic phosphates at 22-23°C (room temperature) and at 37° C was measured with a Beckman spectrophotometer. The measurements relevant to this paper are given in Table 1. Repeated experiments using the same solution showed extremely small variation (see Figs. 13, 17, 31). Experiments performed another day with solutions made up in the same way sometimes showed significant differences in the free phosphate extractable in controls, and occasionally in the amplitude of the phasic changes described. However, the maxima and the minima were always the same, i.e. the shapes of the graphs were always the same, and values for two samples of the same solution done consecutively can be seen on the graphs to be very close together. At the end of a day's experiment, all the test tubes were read randomly, without the experimenter knowing the code. This was only revealed after the individual samples had been measured. The variation in the quantity of phosphate liberated in the controls of the PCr experiments was attributed to the lability of PCr in acid molybdate conditions, especially at 37°C (for review, see Morrison and Ennor, 1960).

Wave length (mμ)	22°C			37°C	
	ATP	PCr	PA	ATP	PCr
330	0.002	0.002	0.017	0.001	0.009
350	0.002	0.001	0.018	0.009	0.007
375	0.002	0.000	0.017	0.009	0.007
400	0.001	0.000	0.016	0.011	0.007
425	0.000	0.001	0.016	0.010	0.006
450	0.002	0.001	0.014	0.010	0.006
475	0.001	0.000	0.014	0.010	0.006
500	0.000	0.000	0.014	0.0010	0.004
525	0.000	-0.001	0.014	0.008	0.004
550	0.000	-0.001	0.013	0.007	0.004
575	0.000	0.001	0.013	0.006	0.004
600	-0.001	0.000	0.013	0.006	0.002
625	0.000	0.000	0.012	0.004	0.003
650	0.000	-0.001	0.012	0.004	0.001
675	0.000	0.001	0.012	0.002	0.001
700	0.000	0.000	0.012	0.002	0.000
725	0.000	0.000	0.012	0.002	0.000
750	0.000	0.000	0.011	0.002	0.000
800	0.000	0.000	0.010	0.002	0.000

Table 1. Optical density of 2 mM solutions of ATP and PCr at 37°C, and PA at 22°C. The ATP and PCr were prepared as described in Methods, shaken gently in the cuvettes, then read against water in a Beckman spectrophotometer, first at 37°C, and the same solution in the same cuvette at 22°C, then again at 37°C. The PA solution was only read al 22°C. The values are the means of each reading done 4 times.

Results

Light

Whereas at 22°C neither ATP, nor PCr solutions had measurable absorption of light at wavelengths of 330-800 mμ, at 37°C, they both absorbed a small, but significant quantities of light, less at the higher wave lengths used (Table 1). PA at 22°C absorbed slightly through the visible range. The quantities of inorganic phosphate, which could be extracted in the absence of light or sound from the 2 mM solutions of ATP, PCr or PA, every 1 min for 10 min, and every 10 sec for 100 sec, showed no significant trends (Figs. l, 2).

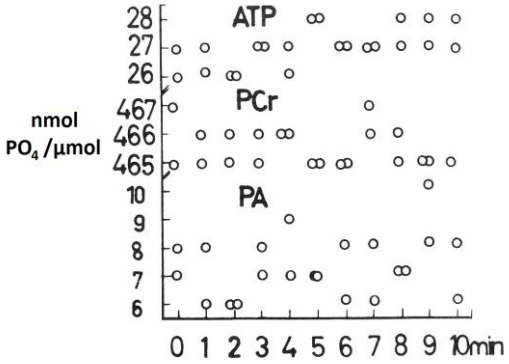

Fig. 1. Measurement of phosphate extractable every 1 min from ATP and PCr at 37°C, and PA at 22°C, *without* application of light, sound, electromagnetic field or centrifugation. In all figures, 0.5 ml of buffered 2 mM ATP, PCr at 37°C or PA at 22°C were kept in the dark for at least 20 min before experiments. In this and all other figures, the two values for each point represent the values obtained by two people doing precisely the same experiment simultaneously.

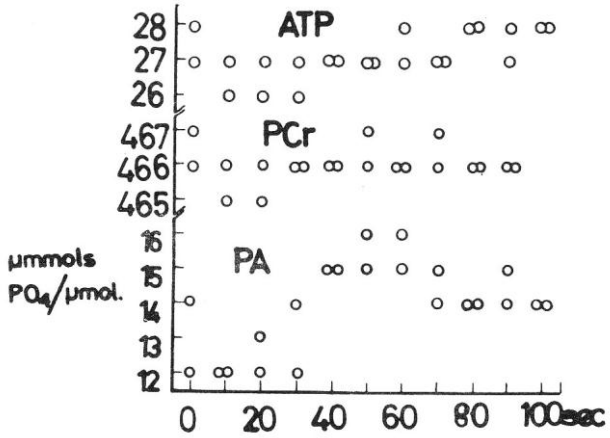

Fig. 2. Measurement of phosphate extractable every 10 sec from ATP, PCr at 37°C and PA at 22°C.

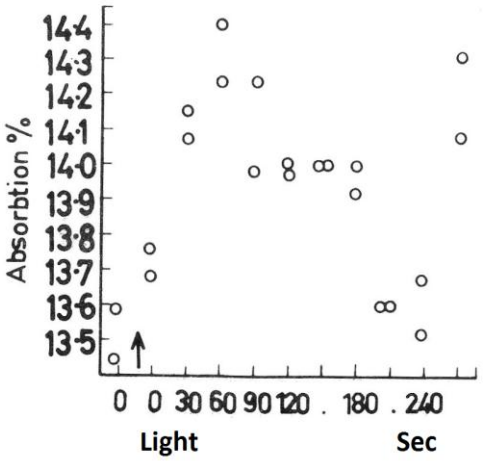

Fig. 3. Effect of 2 sec illumination with laboratory lamplight of 500 lux at 37°C (arrow) on the absorption of 0.5 ml of 2 mM ATP. 1.5 ml of H_20 at 0^{o}**C** was added to controls, immediately after, and every 30 sec subsequently. 2ml of H_20 was added at 22°C to read solutions at 260mμ on the spectrophotometer.

All the other samples were exposed to 500 luxes of laboratory lamp for 2 sec, and 1.5 ml of water added to each pair, every 30 sec. and they were put into ice. They had to be diluted by a further 2 ml of water, to measure their absorption at 260 mμ. It can be seen that immediately after the light, the absorption increased slightly, but 30 sec later it had increased by 0.5%, and at 1 min the maximum increase was 0.8%. This rise in absorption gradually reverted and had returned to the previous level in 210 sec to rise again by 300 sec (Fig.3).

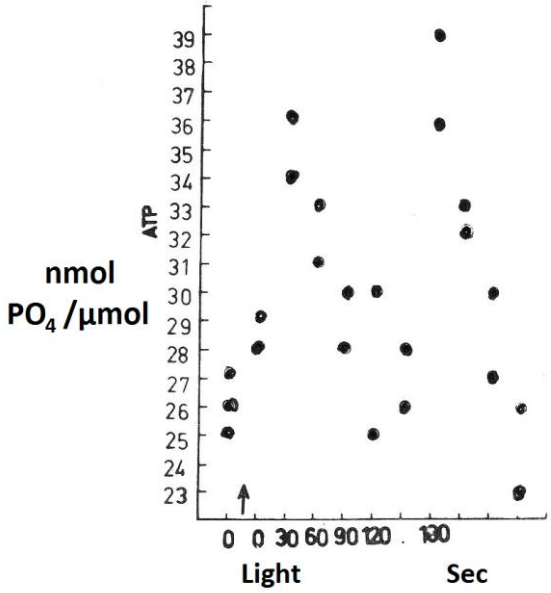

Fig. 4. Effect on ATP of 2 sec illumination of 500 lux of laboratory lamp light (arrow) on the quantity of phosphate subsequently extractable every 30 sec at 37°C.

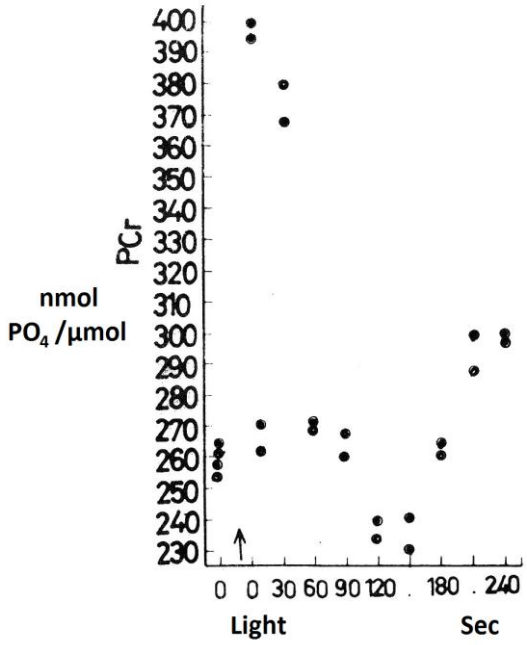

Fig. 5. Effect on PCr of 2 sec illumination of 500 lux of laboratory lamplight on the quantity of phosphate subsequently extractable every 30 sec at 37°C.

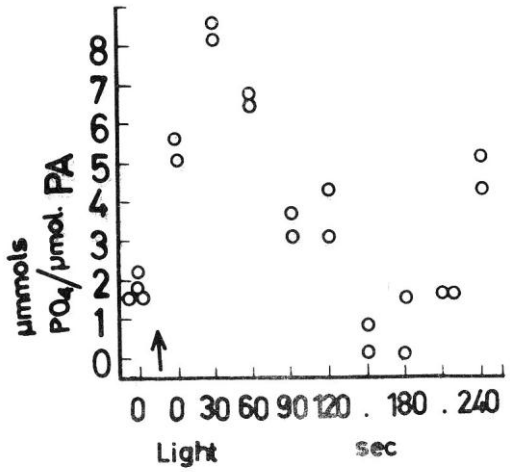

Fig. 6. Effect on PA of 2 sec illumination of 500 lux of laboratory lamplight on quantity of phosphate subsequently extractable every 30 sec at 22°C.

If, instead of measuring absorption after 2 sec light, the quantity of inorganic phosphate was measured, from ATP, it also showed a phasic behaviour. Within 2 sec of 500 luxes of light, the extractable $P0_4$ had risen slightly from its level of 26 nmol/µmole of ATP, but 30 sec later, it had risen to about 36 nmoles; 90-150 sec later it had fallen to its original value, but rose again without further stimulus to a mean of 38 nmoles, 30 sec later. The maximum change in free $P0_4$ was about 15 nmoles from 1 µmole of ATP (Fig. 4). The

quantity of inorganic phosphate extractable from PCr after 2 seconds light rose by 14 nmoles/µmole. It showed a decreased quantity of extractable $P0_4$, 120-150 sec after light (Fig.5). PA at 22°C was also sensitive to 2 sec light, showing the same phasic behaviour, although the maximum quantity of inorganic $P0_4$ was only about 6 nmols/µmol PA(Fig.6). It should be noted that each of these organic phosphates liberates different quantities of $P0_4$ after the same light stimulus, and each follows a specific pattern with time.

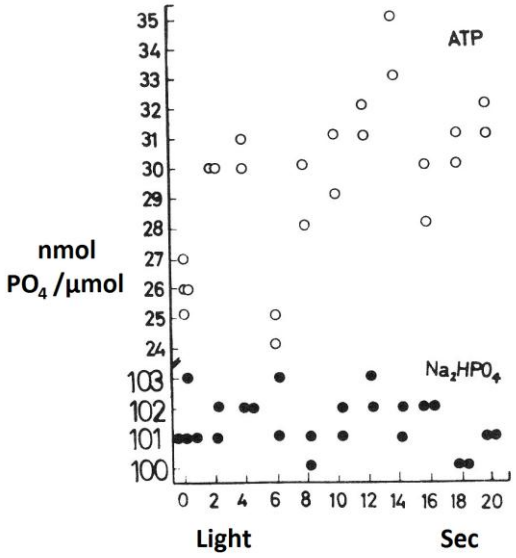

Fig. 7. Exposure of ATP and Na_2HPO_4 (0.1mM) to 500 lux of laboratory lamplight for 0-20 sec at 37°C. The Na_2HPO4 was a control.

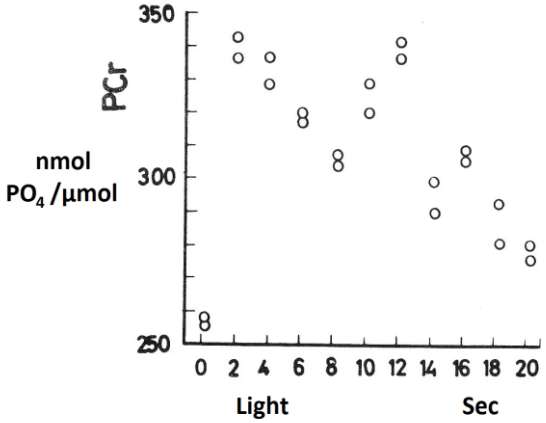

Fig. 8. Exposure of PCr to 500 lux of laboratory lamplight for 0 - 20 sec at 37°C. For other conditions, see Fig. 1.

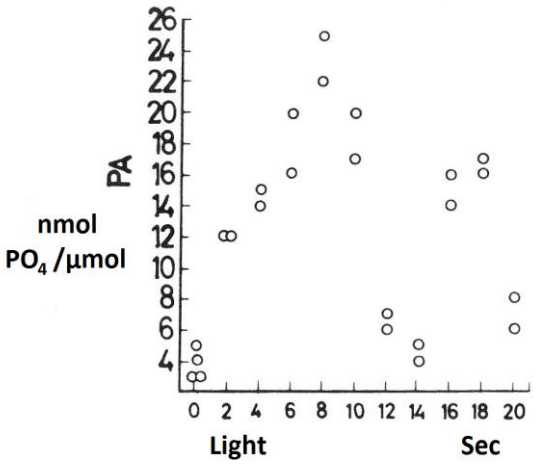

Fig. 9. Exposure of PA to 500 lux of laboratory lamplight for 0 - 20 sec at 22°C.

Examination of the quantity of free phosphate liberated immediately after 2- 20 sec exposure to 500 luxes of laboratory lamp light revealed the same phasic behaviour (Fig.7, 8, 9). ATP after 2,14 and 20 sec exposure, PCr after 20 sec, and PA after 8 and 16 sec, showed peaks of phosphate liberation. ATP after 6 sec, PCr after 20 sec, and PA after 14 sec liberated not significantly more phosphate than could be extracted in the controls in the dark. The quantities of PO_4 extractable from the 0.1µmols of Na_2HPO_4 (Fig. 7) did not show significant differences from the controls (cf. Figs 1 and 2).

Light for 30 sec was increased from 0 to1000 lux, by bringing the lamp nearer. ATP is seen to be maximally affected at 300 luxes, PCr at 600 luxes, and PA at 300 luxes and 600 luxes. On either side of these peaks, the phosphate bond was less easy to break. ATP, exposed to 1000 luxes, and PA under 100-200 luxes, and 400-600 luxes, showed no increase of extractable $P0_4$ (Figs. 10, 11, 12).

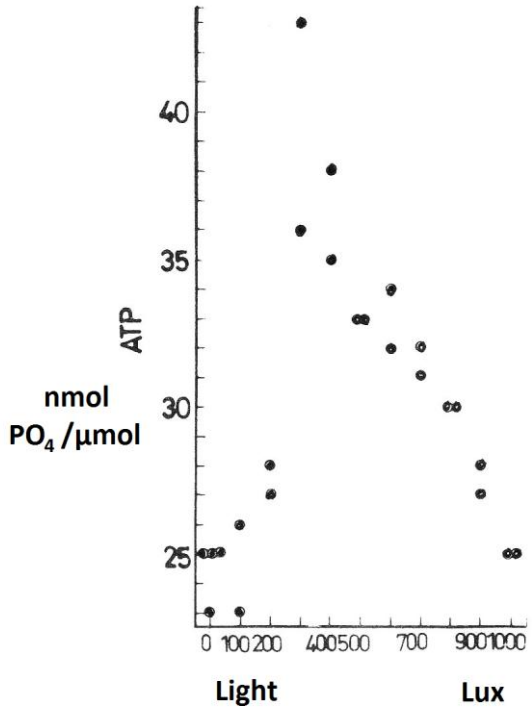

Fig. 10. Exposure of ATP to 0-1000 lux of laboratory lamplight for 30 sec at 37°C. For other conditions, see Fig.1.

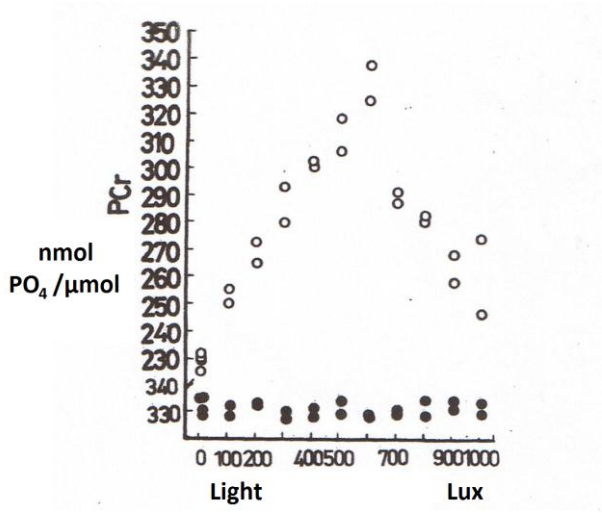

Fig.11. Exposure of PCr and Na_2HPO_4 (0.1mM) to 0 - 1000 lux of laboratory lamplight for 30 sec at 37°C. For other conditions, see Fig. 1.

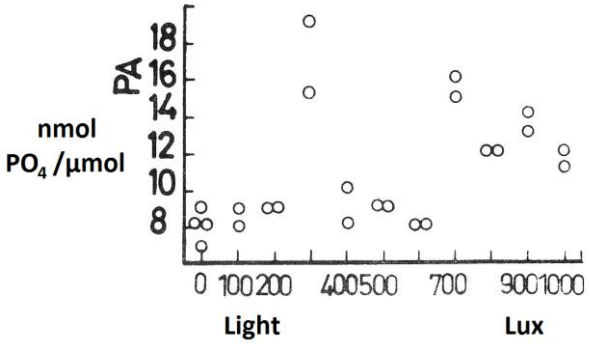

Fig. 12. Exposure of PA to 0-1000 lux of laboratory lamplight for 30 sec at 22°C. For other conditions, see Fig. 1.

An Osram high pressure Hg lamp had peaks of energy output in the visible range at wavelengths of mμ, 313, 333, 365, 403, 435, 480, 547 and 578. These peak values were used as readings for wavelengths. However, each setting thus represented a much different band-width. Exposure of pairs of tubes for 30 sec to each of the coloured lights defined by these conditions showed that different quantities of free $P0_4$ were extractable with the wave-lengths used (Figs 13, 14 and 15). They were also extracted in the dark.

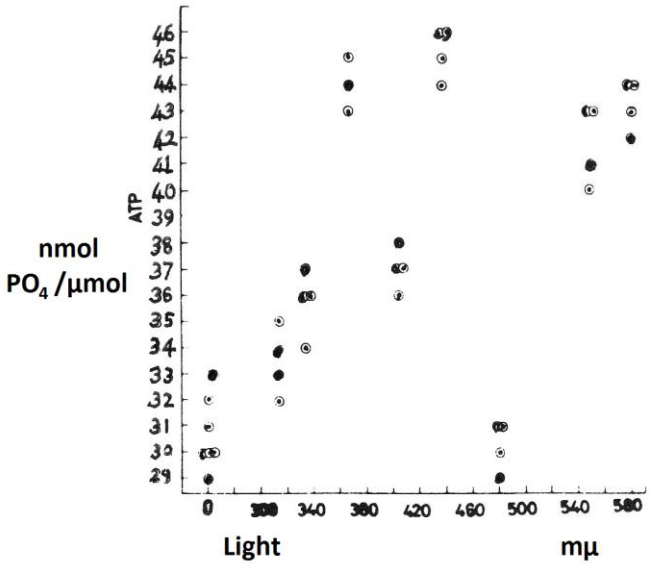

Fig. 13. Exposure of ATP to Osram Hg high pressure lamp light of different wave lengths for 30 seconds at 37°C. Circles with black spots represent a second experiment under the same conditions to show the variation between the experiments. For other conditions, see Fig. 1.

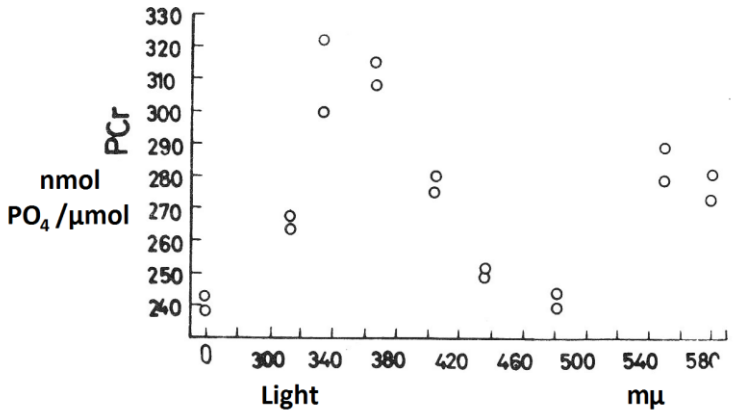

Fig. 14. Exposure of PCr to Osram Hg high pressure lamplight of different wave length maxima for 30 sec at 37°C. For other conditions, see Fig. 1.

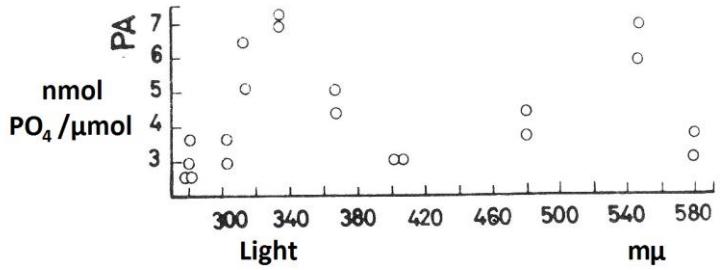

Fig. 15. Exposure of PA to Osram Hg high pressure lamplight of different wave length maxima for 30 sec at 22°C. For other conditions, see Fig. 1.

None of the organic phosphates appeared to be sensitive to the light at 403 mµ, and PA not at 313 mµ or 578 mµ (Figs. 13, 14, 15). It is, of course, possible that during the time of 30 sec, the free phosphate extractable was going through a minimal or maximal value, and would have shown a different figure for a different exposure time, Figure 13 shows 2 experiments on consecutive days on the same solution, which had been kept at 4°C to show the variation between experiments. Using the same lighting conditions, Engineer B. Olander measured the amount of light energy received in watts in the position in which the test tubes were usually placed.

Sound

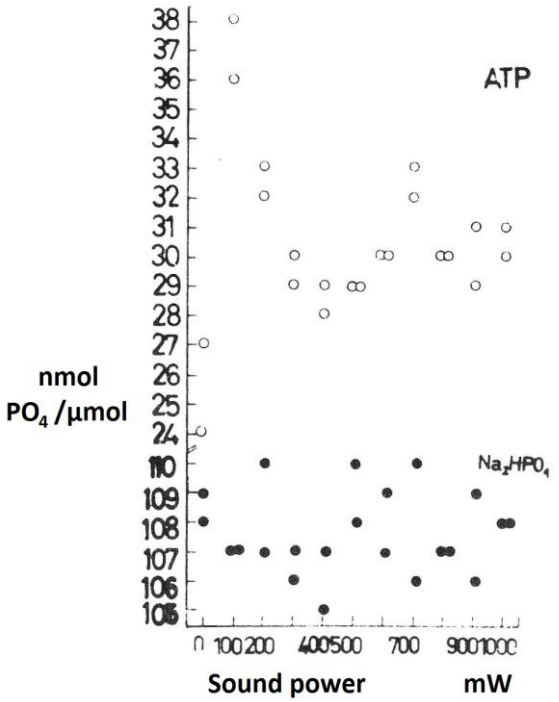

Fig. 16. Exposure of ATP(2mM) and Na_2HPO_4 (0.1 mM) to a loudspeaker 1 metre away with an input of 0-1000 mW at 256 c/s, for 4 sec, at 37°C. This, and all subsequent figures are of experiments done in the dark. For other conditions, see Fig. 1.

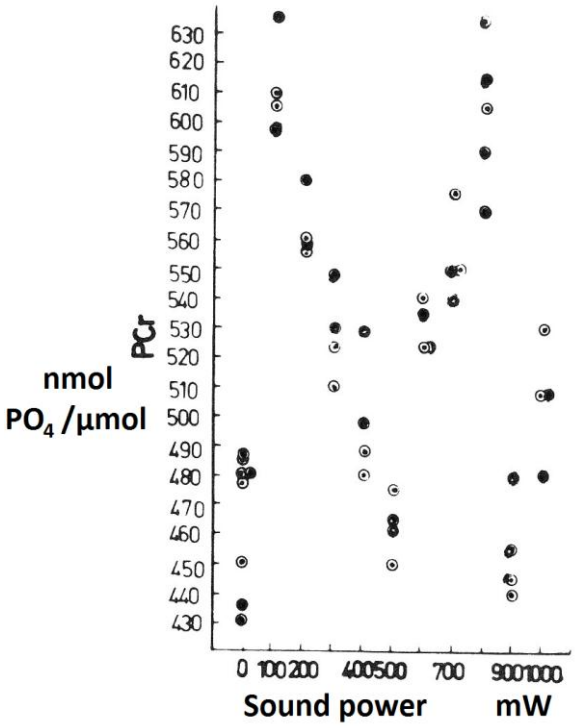

Fig. 17. Exposure of PCr to loudspeaker 1 metre away with an input of 0-1000 mW at 256 c/s for 4 sec in the dark at 37°C. Circles with black spots represent a second experiment to show the variation between experiments on consecutive days.

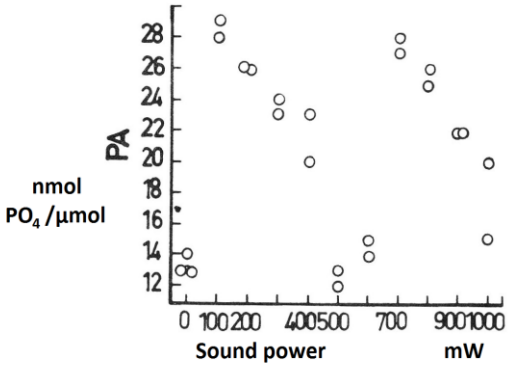

Fig. 18. Exposure of PA to loudspeaker 1 metre away with an input of 0-1000 mW at 56 c/s for 4 sec at 22°C.

A sound of 0-1000 mW into a calibrated linearly rising output loudspeaker 1 metre from all the tube at 256 c/s, was turned on all the tubes for 4 sec for each power. 100 mW and 700-800mW had most effect on ATP, a 100 mW and 800 mW on PCr, and 100 mW and 700 mW on PA. Each of these high energy phosphates also showed intensities of power, which had minimal effects on the high-energy phosphates (Figs. 16, 17, 18). Fig. 16 shows the quantity of inorganic phosphate extractable from Na_2HPO_4 under similar conditions. Repetition of this experiment showed no peaks but the large variation in readings remains. The quantities of inorganic phosphate extracted at the fixed power input to the loudspeaker of 50rnW and different frequencies from 0 to 1000 c/s also showed that the liberation of inorganic phosphate showed a distinct frequency sensitivity (Figs. 19, 20, 21). Fig. 17, shows the variation between 2 experiments done on consecutive days with the same solutions.

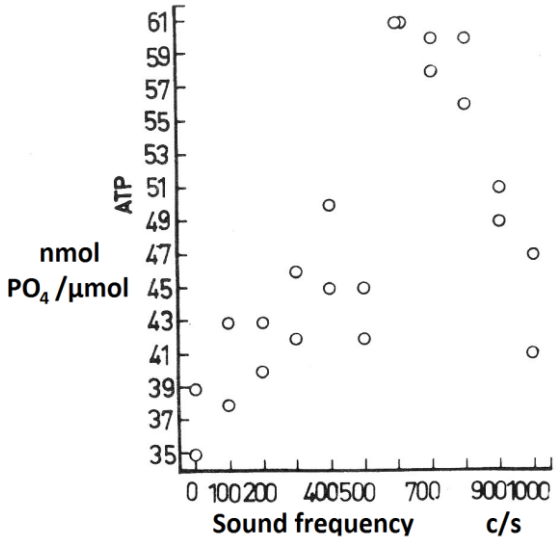

Fig. 19. Exposure of ATP to loudspeaker 1 metre away with constant power output, and frequencies 0 - 1000 c/s for 4 sec at 37°C.

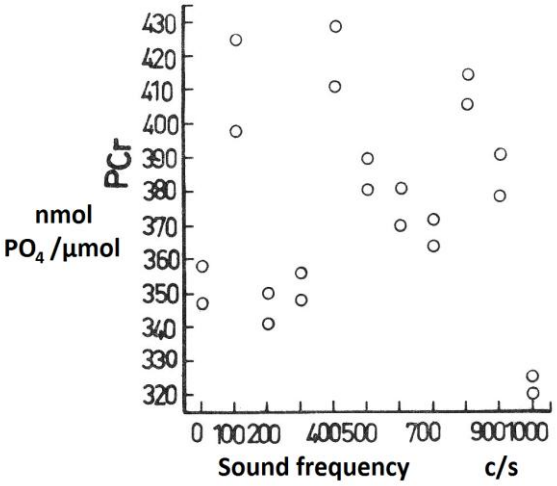

Fig. 20. Exposure of PCr to loudspeaker 1 metre away with constant power output, and frequencies of 0-1000 c/s for 4 sec at 37°C. For other conditions, see Figs. 1, 16.

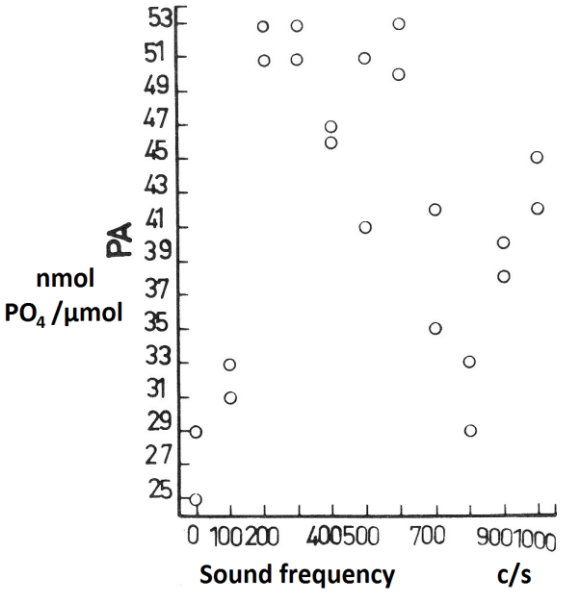

Fig. 21.Exposure of PA to loudspeaker 1 metre away with constant power output, and frequencies of 0 1000 c/s for 4 sec at 22°C. For other conditions, see Fig. 1, 16.

Electric current

Linear increase of a.c. voltage round the coil from 0 to 240 V in 24 V steps showed that all voltages produced some instability. ATP released between about 5 - 9 nmoles of phosphate, except for 60 V which had no effect (Fig. 22). The quantity of inorganic phosphate which was extracted from PCr was decreased between 168 to 240 V below the control value (Fig. 23). PA (upon which only 1 experiment was done) was quite insensitive to 72, 96 and 216 V, but allowed 4-10 nmol to be released by other voltages in the coil (Fig. 24). The current in the coil released phosphate from ATP and PCr in an oscillatory manner

for 20 secs (Figs. 25, 26), and non-linearly in respect of voltage applied around the coil (Figs 27, 28, 29). I subsequently concluded that the effects of current in the coil could have been partly due to heating the solutions.

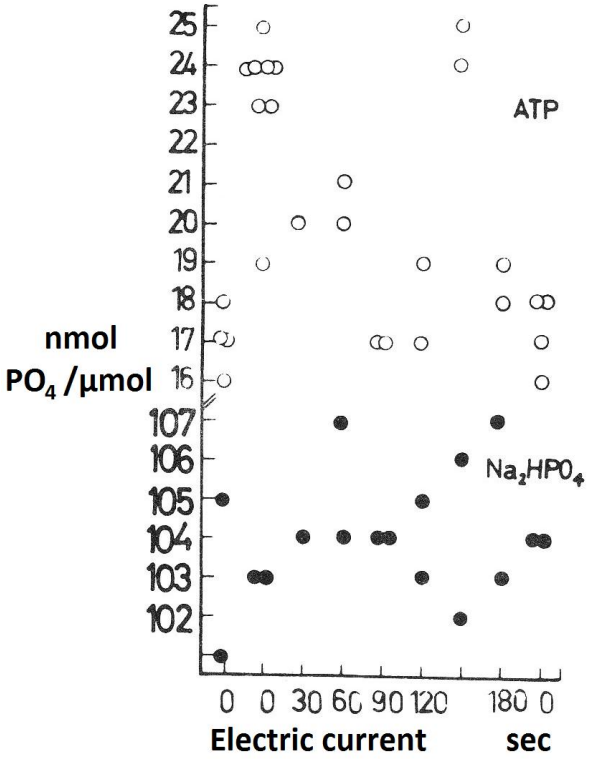

Fig. 22. Effect on ATP and Na_2HPO_4 (0.1mM) of 48 V in coil around the test tubes on subsequent extraction of phosphate. For other conditions, see Figs. 1, 16. Note the large variation in the Na_2HPO_4 experiment, and that controls were taken before and after the electric current was turned on.

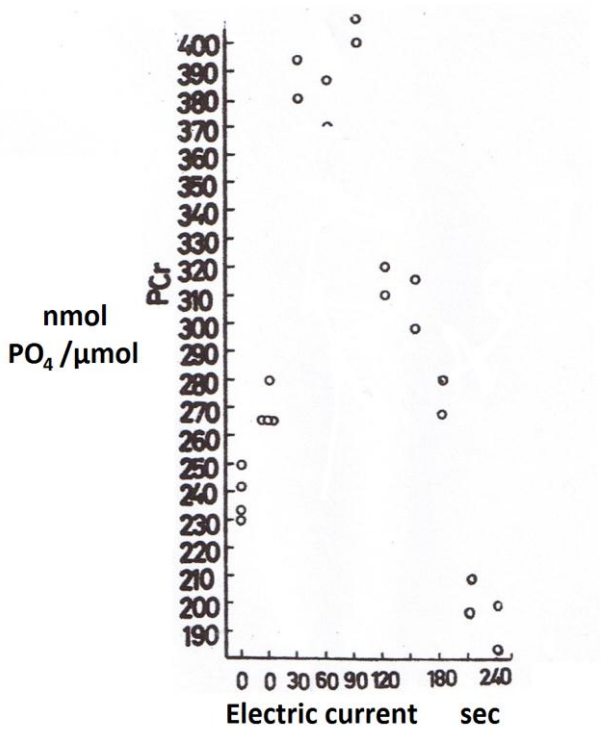

Figure 23. Effect on PCr of 48V in coil around test for 4 seconds at 37°C on subsequent extraction of phosphate. For other conditions, see Figs. 1, 16. Please note that after 210 sec, there is less extraction of phosphate than in controls.

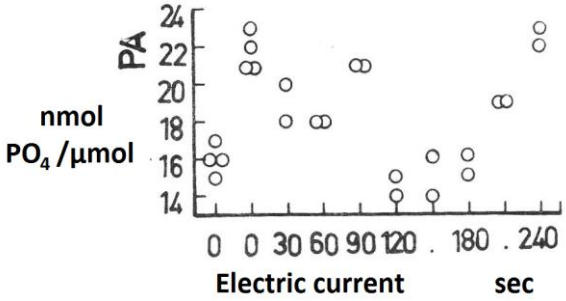

Fig. 24. Effect on PA of 48 V in coil around test tube for 4 sec at 22°C on subsequent extraction of phosphate. For other conditions, see Figs. 1, 16.

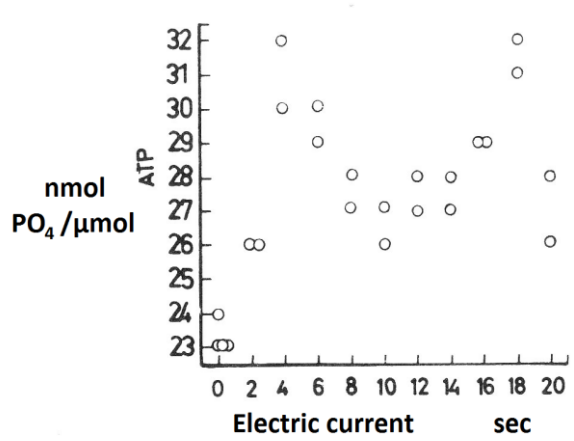

Fig. 25. Effect on ATP of 0 - 20 sec of 48 V in a coil around test tube at 37°. For other conditions, see Figs. 1, 16.

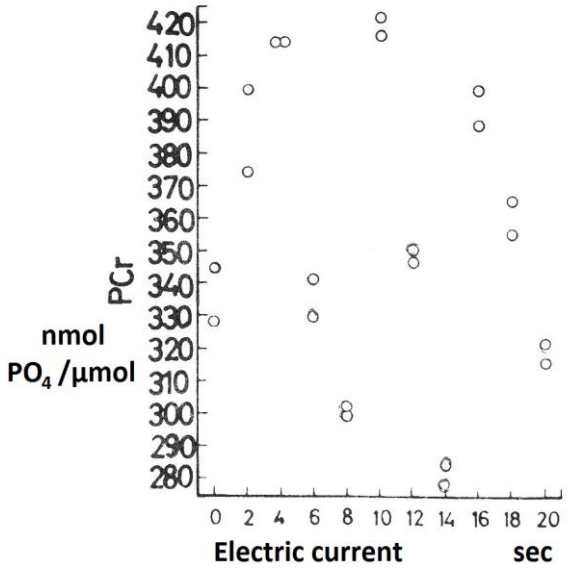

Fig. 26. Effect on PCr of 0-20 sec of 48 V in a coil around test tube at 37^0C. For other conditions, see Figs 1, 16.

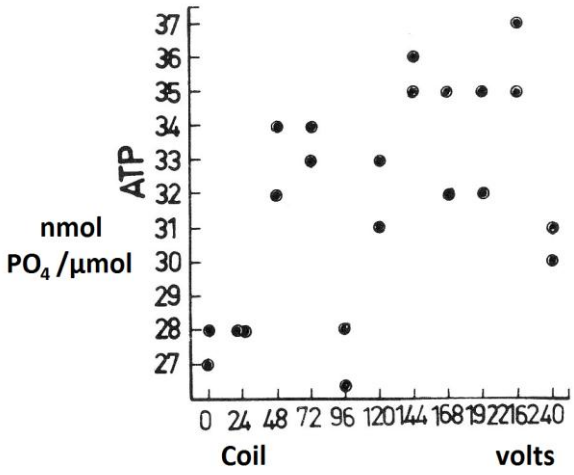

Fig. 27. Effect on ATP of 0 - 240 V in coil around test tube for 10 sec at 37°C. For other conditions, see Figs. 1, 16.

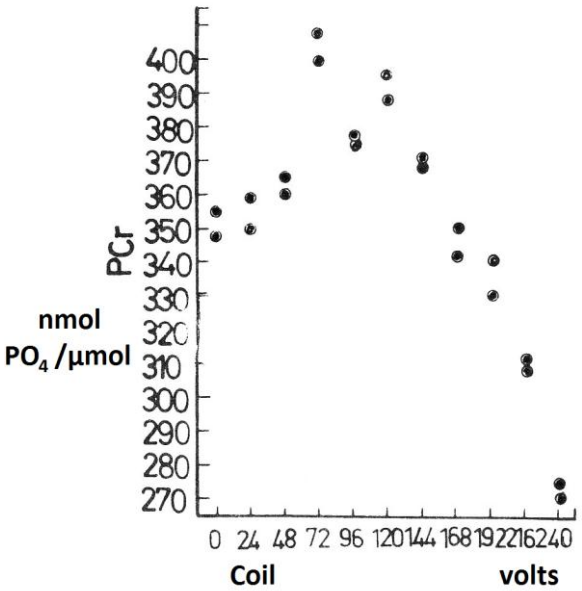

Fig. 28. Effect on PCr of 0 - 240 V in coil around test tube for 10 sec at 37°C. For other conditions, see Figs. 1, 16. Note that above 192 V, less phosphate was extracted than from the controls.

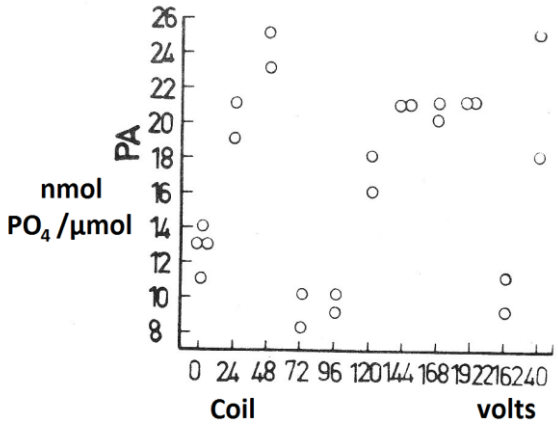

Fig. 29. Effect on PA of 0 - 240 V in coil around test tube for 10 sec at 22°C. For other conditions, see Figs. 1, 16.

Centrifugation

Turning on the centrifuge for 1 min to a setting of 1000 rev/mm, leaving it to stop for 60 sec, and then extracting it, showed considerably increased quantities of phosphate extractable from ATP, PCr and PA, 60 sec after the end of centrifugation. In the cases of ATP and PCr it rose for a further 30 sec, and then gradually declined in minutes (Figs. 30, 31, 32). The PA showed a phasic response, being at the control value after 150 sec, and much lower at 240 sec (Fig. 32).

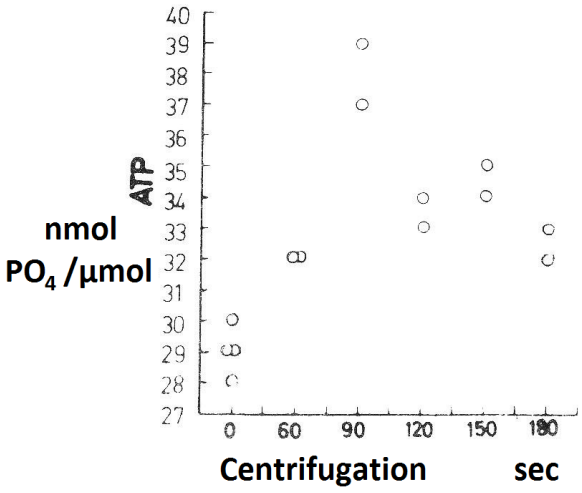

Fig. 30. Effect on ATP of centrifugation, 1000 rev/min, for 1 min on subsequent extraction of phosphate at 37°C. The centrifuge took 50- 55 sec to stop, and the first extraction was done at 60 sec.

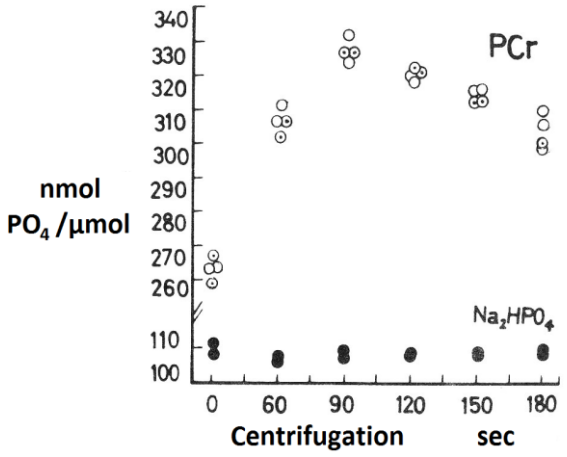

Fig. 31. Effect on PCr (2mM), (upper), and Na_2HPO_4 (0.1 mM), (lower), of centrifugation, at 1000 rev/mm, for 1 min on subsequent extraction of phosphate at 37°C. Two experiments are superimposed. For other conditions, see Figs. 1, 16, 30.When only one value appears, it was because the two values found were exactly the same.

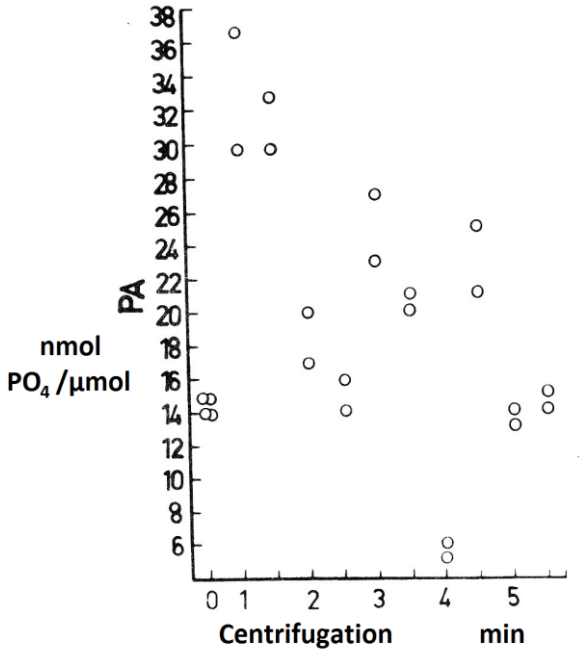

Fig. 32. Effect on PA of centrifugation, at 1000 rev/mm for 1 min on subsequent extraction of phosphate at 22°C. For other conditions, see Figs. 1, 16, 30.

Sodium and potassium ion concentrations

Keeping 1 µmo1 of ATP at 37°C in the dark for 30 mm in the presence of 0 - 150 mM of Na^+ or K^+ solutions in 10 steps shows that between the ranges 10 and 30 mM, 40 and 90

mM and 100- 150 mM Na^+, the quantity of inorganic phosphate measured was inversely proportional to the increasing concentration of Na^+ with very steep slopes; it was apparently independent of the K^+ concentration (Fig. 33). PCr and PA were both insensitive to the K^+ concentrations also, but PCr released phosphate at concentrations above 50 mM Na^+, while PA released it only below 60 mM(Fig. 35). The effect of mixing the Na^+ and K^+, so that together they made up 150 mM, was in all cases to increase the quantity of phosphate liberated (Figs. 36, 37, 38). With Na^+ plus K^+ together, the amount of phosphate liberated by concentrations between 100 and 150 mM Na^+ was unchanged by the presence of K^+, but between 90 and 50 mM Na, the slope of the phosphate liberated from ATP was reversed (cf. Fig. 33, 36). The presence of 0.1 mM ouabain had no effect on the liberation of phosphate by ATP. The presence of K^+ with Na+ increased the phosphate liberated from PCr at all concentrations, and actually made the PCr sensitive to 10- 50 mM Na^+ to which it was not previously sensitive (cf. Figs. 34, 37). Fig. 37 shows two similar experiments to show the variation. Mixing the Na^+ and K^+ had the greatest effect on PA at 22°C. Between 30 and 80 mM Na^+ in the mixed solution permitted the extraction of twice the amount of inorganic phosphate as any of the Na^+ containing solutions by themselves (cf. Figs. 35, 38). There was no significant difference between the quantities or phosphate extracted from Na_2HPO_4 in the presence of the different Na^+ plus K^+ mixtures (shown below Fig. 33).

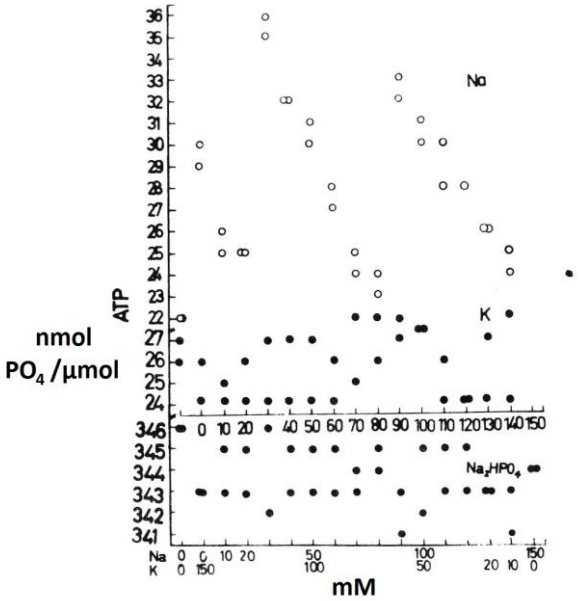

Fig. 33. Effect on ATP of different concentrations of Na^+, (top) K^+, (middle) on the extraction of phosphate in the dark at 37°C. Lowest graph, Na^+ plus K^+, on Na_2HPO_4 (0.1 mM) is the control for Figs. 34, 35, 36, but is put here for convenience. 00 in the abscissa is 150mM sucrose.

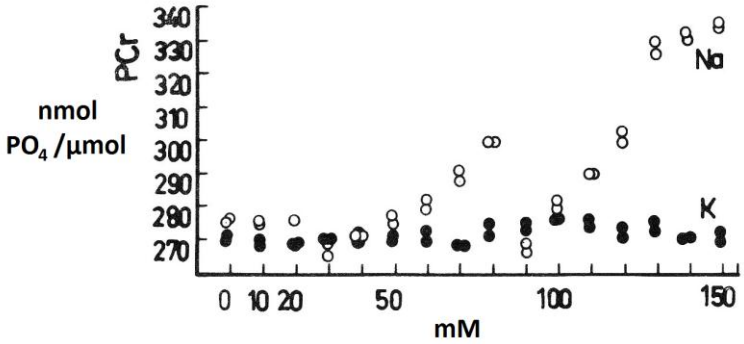

Fig. 34. Effect on PCr different concentrations of Na^+ extraction of phosphate in dark at 37°C.

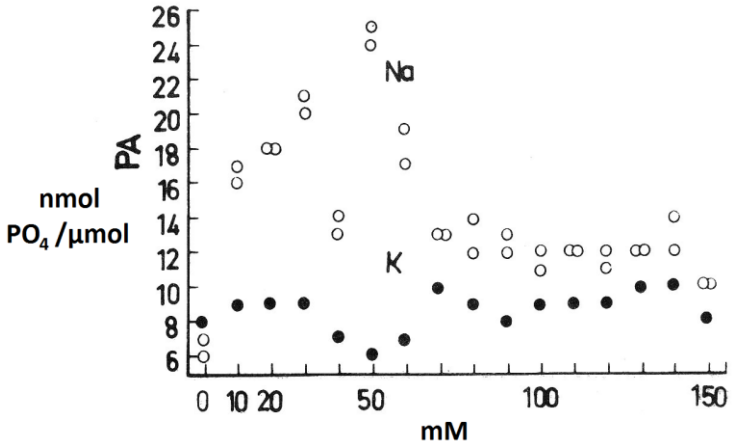

Fig. 35. Effect on PA of different concentrations of Na^+ (open circles), K^+ (closed circles), on extraction of phosphate in the dark at 22°C. Insufficient PA was available for duplicates of the K+ experiment.

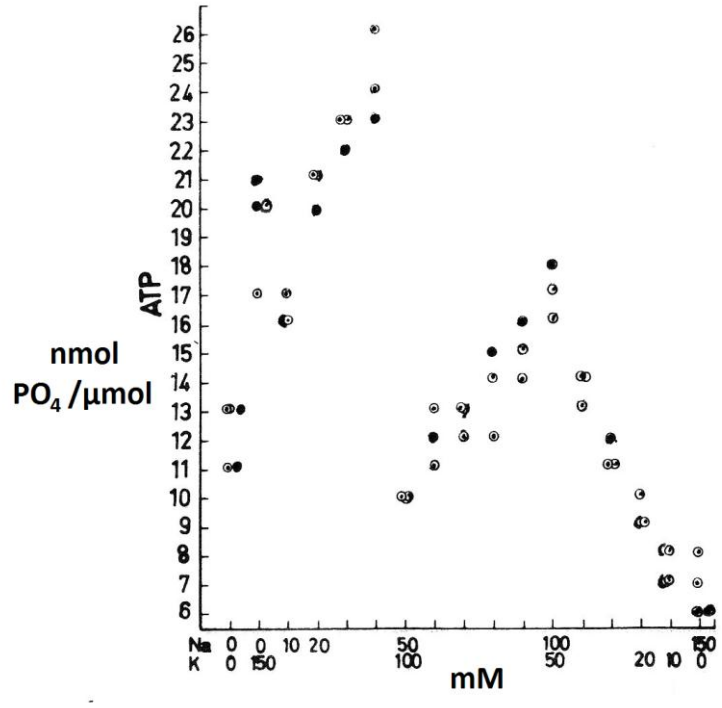

Fig. 36. Effect on ATP of different mixtures of Na^+ and K^+ totalling 150 mM, in the absence of (open circles), and presence of (circles with spots), 0.1 mM ouabain, on the extraction of phosphate in the dark at 37°C. 00 is the control with 150 mM sucrose.

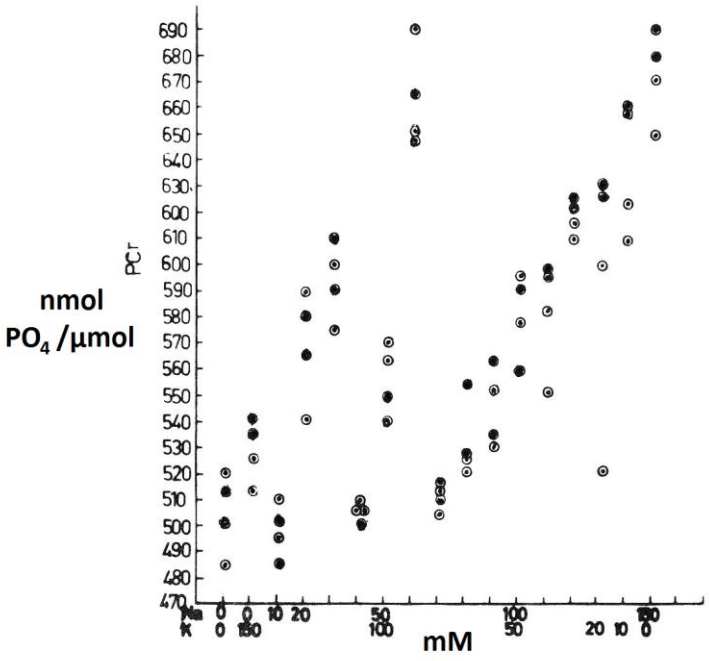

Fig. 37. Effect on PCr of different mixtures of Na^+ plus K^+ totalling 150 mM on the extraction of phosphate in the dark at 37°C. 00 is control with 150 mM sucrose. 2 consecutive experiments are superimposed.

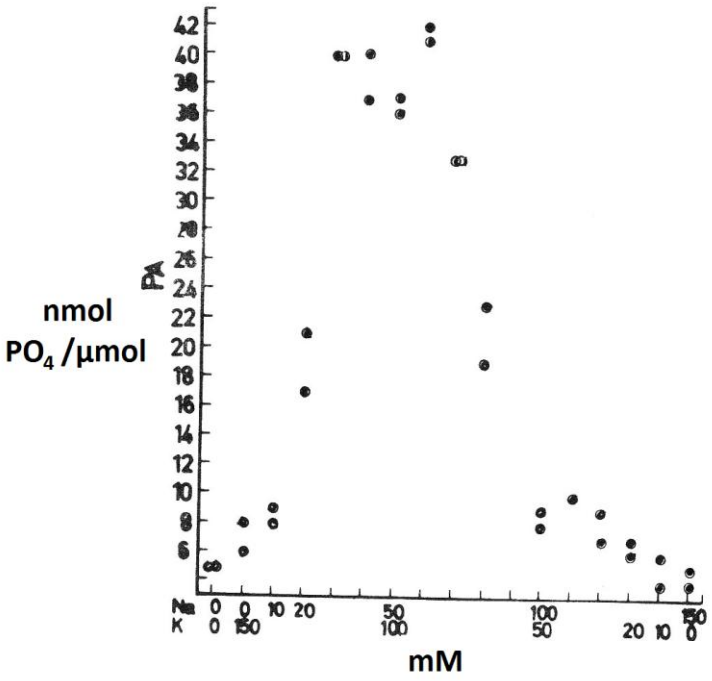

Fig. 38. Effect on PA of different mixtures of Na^+ plus K^+ totalling 150 mM on the extraction of phosphate in the dark at 22°C.00 in the abscissa is the control with 150 mM sucrose.

Discussion

Transducer properties

ATP and PCr at 37^0C and PA at 22^0C are organic phosphates, whose terminal phosphates could be released in greater or less quantities when they were subjected to light, sound, electrical currents, centrifugation or different sodium concentrations. Light, sound and electrical currents induced a phasic change in the quantity of inorganic phosphate extractable, after the cessation of the stimulus. The particular quantity released was a function of the time for which the stimulus had been given, the power of the stimulus, and its frequency. The sensitivity of the system had maxima and minima. Although most of the methods of applying energy caused more inorganic phosphate to be released, some (e.g. more than 168 V applied round the coil (Fig. 28)) decreased the quantity released. Since the three organic phosphates did not change significantly for 100 sec or 10 mm, (Fig. 1, 2). without the application of particular stimuli, and since the quantity of phosphate measured in Na_2HPO_4 solution did not vary significantly as a result of the same agents, and since these three phosphates liberate relatively large quantities of chemical energy on their hydrolysis, (Lipmann. 1941; Atkinson and Morton, 1960), it follows that they could act as transducers. The concentrations, 2mM, of ATP and PCr were chosen to be at the same order as those found in the whole mammalian brain (see Heald, 1960), and PA was used in the same concentration. Furthermore the concentrations of Na^+ and K^+ studied were approximately those found within the living mammalian cell and changing over the range over which they might change. The normal ranges of Na^+ and K^+ of the inside of the cell in poikilotherms which would be relevant to the effects of these ions on PA were not studied in the present experiments.

In the experiments on exposure of the solutions to 2 secs of light, 4 secs of current, or 1 min of centrifugation, it is seen that the quantity of inorganic phosphate extractable *after* the stimulus would return to its previous level, if it were not extracted. Thus the applied agent would *not split off the inorganic phosphate itself*, it would render it more or less easy to extract; in the case of the present experiments it was the shaking prior to extraction which released the phosphate. If another agent was not operative, the phosphate energy would not be released. The effect of the agent appears to be as if it induced a phasic instability of the terminal phosphate bond.

One possible explanation for these phenomena is that there is a reversible oxidation - reduction system. In recent experiments, to be submitted for publication, the light effects on ATP and PCr shown have been completely abolished by bubbling N_2 for 10 sec through the solutions, and adding $FeSO_4$.

Light

Two kinds of light were used. Firstly, the commercial light source over a normal range of illuminations; secondly, light of known wave length. The first was used to demonstrate the phenomena, and also so that they could be simply repeated. The second was used so that the energy could be measured.

ATP and PCr which have negligible absorption at room temperature, have a slight but measurable absorption at 37°C, (Table 1), the absorption has been shown to go up in ATP on its illumination (figs 3-13). Examination of the absorption of other compounds of biological interest at 37°C and at neutral pH might well show absorption not seen at 22°C.

Since the hydrolyses liberate so much energy, it is not thermodynamically possible that they would reverse without another energy source. Therefore, the finding that *after* subjection to light, the quantity of phosphate recovered increased, then reverted to normal

(Fig. 3, 4, 5), meant that the light did not split the phosphate bond, it only caused it to weaken and strengthen. In the situation in which the bond was weakened, it would break more easily. If it was not broken at this time, it would then strengthen.

These results may explain those of Wells and Johnson (1953) who found that visible light liberated phosphates from ATP. Shugar (1960) in reviewing the results of such experiments on DPN pointed out that it does not absorb visible light, and even the UV from a light source would be filtered out by glass. If ATP does not absorb light, according to the 1st law of photochemistry, it cannot produce an effect. It seems possible that in Wells and Johnson's experiments, the light source was powerful enough to warm the solution. The conflicting results of the effects of visible light on nucleotides (for review, see Shugar, 1960) could be due to the phasic nature of the change induced. Garay and Guba (1953) reported breakdown of ATP with UV light which "reversed" in minutes. They did not test visible light. The present results suggest that values for the "high energy" phosphates of incubated tissue which usually has been shaken at 37°C, while exposed to a probable illumination of 200- 400 lux in the laboratory would be underestimated. They reproduce the conditions in which the inorganic phosphate here has been shown to rise. It is also worth noting that adrenalin, scintillation fluids, ether and lactate, are all affected by light, although they are not coloured solutions.

The peak effects at particular intensities of illumination on ATP, PCr and PA (Fig. 10, 11. 12) suggest that the phosphate bond of these phosphates, but not Na_2HPO_4 could be rendered unstable by particular quantities of energy. Visible light caused relaxation of rabbit aorta muscle in vitro (Furchgott, Ehrenreich and Greenblatt, 1961). The muscle was in a medium at 37°C; it required oxygen, and the effectiveness of the light increased with wavelengths below 450 mμ. The authors estimated that if correction could be made for internal filtering, the light would be effective down to 250 mμ. The contraction may be mediated through PCr.

Sound

The present experiments appear to be the first to report chemical effects of domestic levels of sound. The sensitivity to sound was first noticed when the levels of phosphate extracted fluctuated as a result of speech. Besides the pure sounds tested, it was also shown that normal speech (4 experiments) and playing of recorded bagpipes from a tape recorder (two experiments), both had the same effect, but these experiments have not been included here since we did not quantify the power of the sound.

After 4 sec sound with 50 mW at 1000 c/s, the level of phosphate extractable from PCr was even lower than the control (Fig. 20). Likewise with more than 194 V in the coil inducing an electromagnetic field in the PCr, the extractable phosphate was less than in the controls (Fig. 28). This would be expected if one imagines as a model of the terminal phosphate bond a spring in the neutral position. At certain phases, it would pass through the neutral position, so that the phosphate radical would be more strongly held to the rest of the molecule. An implication of these experiments is that 'high energy' phosphates should be measured either in a quiet room, or not being shaken. In the latter case, although the phosphate would be activated, it would return to its previous level, in the dark. It might be expected that the three phosphates would have measurable sound absorption in an acoustic spectrometer.

Electric current

The electromagnetic field induced was shown to activate these phosphates. The technique was designed to be both simple, and without electrodes (as well as cheap). It was not possible to measure the current induced in the solution, as its resistance was not known. However, it could not have been high enough itself to hydrolyse the phosphates, or to electrolyse them, as in all the experiments with electric current (Figs. 22- 29, except Fig, 25) there were some conditions in which no more than the control quantity of inorganic phosphate could be extracted. The fact that in Figs. 22 -24, where current was applied for 4 seconds, the phasic change of phosphate extractable goes through zero means that the 'recovery' of high energy phosphates in cerebral slices, for example, after their fall following electrical stimulation (Heald, 1954) may be due to the loss of activation, not resynthesis of the phosphates.

The greater variation in the extractability of phosphate from Na_2HPO_4 during 0- 20 sec of electric current and sound 0 - 1000 mW (cf, Figs. 7, 16 and 22) could well mask a slight or very rapid effect of this agent on this phosphate bond. This may require very precise and rapid measurement to demonstrate.

It is possible that the electromagnetic effect was partly due to heating.

Centrifugation

The finding that centrifugation at 37°C itself activates the phosphate (Figs. 31, 32, 33) (the ATP and PCr with a half time of 3-4 min) suggests that the very slight shearing of the phosphate bond was enough to loosen it. Any further treatment within about 10 min might well break down the 'high energy' phosphate. Nyman and Whittaker (1963) reported a considerable loss of ATP from particulate fractions, however mildly they were prepared.

Na^+ and K^+

The presence of the K^+ in the mixture gave higher values for the phosphate extracted with the Na^+ (Figs 36, 37 and 38). The maximum quantity of phosphate split off was calculated at about 1.4 %, 16% and 3.6% of the total terminal phosphate of ATP, PCr and PA, respectively. The Na^+ and K^+ concentrations chosen were those which would occur inside the cell, during the action potential, represented going from left to right in the figures. In the present experiments a change of 10 - 30 mM concentration of Na+, in the presence of K^+, would result in the liberation of 9 nmoles of phosphate from 1μmole of ATP, and 90 nmoles of PCr. Phosphates are present in nervous tissues at least 2 - 3 times this concentration,(Heald, 1960)..

The Na^+ and K^+ experiments differ from the others in that no activation is studied here. The phosphate was measured only in the presence of these ion concentrations. Lowenstein (1960) using a much higher pH examined the transphosphorylation from ATP. He concluded that it was sensitive to the K^+ but not the Na^+ concentration. Thus the stability of the phosphates in the presence of these ions is probably also dependant upon the pH. Although the quantity of phosphate liberated by Na^+ is not large, shaking the solution in the light with sound in the laboratory would probable release even more. Experiments in which phosphatases are estimated by the amount of inorganic phosphate liberated, especially when demonstrating Na^+ and K^+ activation (e.g.Skou, 1957) should have, as controls, the substrate in the presence of the ions equivalent to that of the final concentration found in the experimental samples.

General implications of the present experiments

It is further possible that these properties are shared by other esters of ions of nitrogen, oxygen and sulphur, all of which have unshaired pairs of electrons (Kasha, 1950). Reid (1957) gives a list of 16 groups of molecules in which such transitions have been characterised. Among these are aldehydes, ketones, esters, pyridines, and pyrimidines. If some of these compounds of biological interest do exhibit such instability, especially so strikingly differently in the case of ATP and PCr at 37°C compared with 22°C, considerable care must be taken in the choice of control conditions of substrates in measurement of enzyme activities.

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The sensitivity of these phosphates to light, sound, electric currents, and centrifugation, is paralleled by the existence of receptors to all these modalities (for reviews, see Granit, 1955; Bullock, 1959). The phasic nature of the present changes have analogies both in the refractory period of nervous activity, and in the adaptation of sensory receptors to continuous stimuli. The latter is seen in the experiments where 0-20 sec light, sound or electric stimuli were given. The longer stimulus did not seem to engender a greater reaction.

Summary

1. At pH 7.3- 7.5, 2 mM solution of ATP and PCr at 37^0C, like PA at 22°C absorbed visible light (350— 625 mμ),slightly.

2. Visible light of 500 lux for 2 sec caused a periodic increase for minutes in absorbtion of ATP at 260 mμ.

3. Such light also caused a similar periodic rise and fall of quantity of extractable inorganic phosphate from ATP, PCr and PA. The quantity extractable depended non-linearly on the time of exposure to such light (0- 20 sec), its intensity between 100 and 1000 lux, and its wavelength.

4. The release of inorganic phosphate depended non-linearly on the energy output and frequency of sound between 100 and 1000 mW at 256 c/s input, and 100 and 1000 c/s at 50 mW output, of a loudspeaker 1 metre away.

5. An electric current activated the solutions for minutes. The quantity of phosphate released was also non-linearly sensitive to the time of the current and its voltage.

6. Centrifugation for 1 min at 1000 rev/min activated ATP, PCr and PA for at least 3 min subsequently.

7. The quantity of extractable phosphate was dependent on the Na^+ concentration between 10 and 150 mM, apparently unaffected by the K^+, but Na^+ plus K^+ had greater effect than Na^+ alone.

8. There was no significant phasic component in the amount of phosphate released from these substances in the absence of light, sound, centrifugation, electromagnetic field, Na^+ or $K,^+$ on 0.1mM Na_2HPO_4 solutions.

Acknowledgements

I wish to thank Prof. H. Hydén for help and advice, Dr. W. Grampp and Engineer S. Larsson for many useful discussions. Miss A. Bäckman, and Miss I. Augustsson gave me careful and patient technical help. I would also like to thank Dr. A. Ennor for providing the pure phosphoarginine sample, and Engineer B. Olander for measuring the energy from the light source used. The Söderberg Foundation kindly financed this research.

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Note added on the 15th of February, 2012

I submitted the above manuscript to several journals in 1964. It was refused. I felt particularly aggrieved when an abstract was refused publication at a meeting of the (British) Physiological Society in November, 1964, in circumstances, which I have described elsewhere (Hillman, 1991). However, a very brief abstract appeared at the International Union of Biochemistry in New York in 1964, and I also published a paper on the effect of visible light on ATP (Hillman, 1966). I was not able to describe the effects of the other agents on ATP, or any of these other agents on CrP or ArP.

Since none of the solutions contained any sources of energy, other than the 'high energy' phosphates themselves, it was not difficult to understand how light and the other modalities could release *more* inorganic phosphate, but it was difficult to understand how the inorganic phosphate could *recombine* to free less. Therefore, I postulated that there were two forms of ATP, a stable form, which was in equilibrium with another less stable form, ATP*. The latter was broken down by the Berenblum and Chain reagents (1938), when the inorganic phosphate was extracted.

In Moscow in the 1950s, Boris Belousov found that transition metal ions, Ce , Mn, Fe, Ru, Co, Cu, Cr, Ag, Ni, and Os, catalysed the oxidation of organic reductants by bromic acid in acidic solutions. The reactions oscillated slowly and in real time, and the solutions changed reversibly from colourless to yellow or violet. Concentric two-dimensional symmetrical shapes formed in these solutions. Sometimes, they resembled endoplasmic reticulum. Belousov had the greatest difficulty in publishing these findings (Belousov, 1959; Winfree, 1984; Epstein and Pojman, 1998). Subsequently, Anatol Zhabotinsky was intrigued by Belousov's experiments. He repeated them (Zhabotinsky, 1964), and they became known as Belousov- Zhabotinsky (BZ) reactions. Zhabotinsky moved to Brandeis University, Waltham, Massachusetts, and, sadly, died there in 2004. The current head of Department there is Professor Irving R. Epstein, who has published extensively on non-equilibrium chemical thermodynamics.

In 1964, I carried out 350 experiments at the Institute of Neurobiology,Göteborg, as described above. Unfortunately, at the time, I did not know of Belousov and Zhabotinsky's work. Recently, in 2012, I wondered about the possible relationships between their and my experiments. One may ask: (i) is it possible that the energy for the original Belousov reactions may have come from, or been catalysed by, the light, sound, mixing, exothermic reactions, or, even the pouring, during the experiments (ii) in preliminary experiments, I also found some effects on the extraction of phosphates from mixtures of 2 mM glucose and 2mM Na_2HPO_4. I did not do enough experiments to publish, but it would be useful to repeat these experiments; (iii) it would be interesting to know if other 'high-energy' phosphates and nucleotides also showed these phenomena; (iv) it would be worthwhile to explore if these effects on ATP, PCr and ArP were examples of non-equilibrium thermodynamics.

In 1964, I sent the above manuscript to the International Information Exchange, no 1, run by the National Institute of Health. It was received on the 27th of July, 1964, and sent out

on the 30th of July, 1964, as Scientific Memo, #190. I noted that Chance (1954), Shnoll (1958) and Hommes (1964) had observed oscillations in the presence of tissue. I wrote to them to ask if they themselves had ever noticed such oscillations in the reagents alone, in the *absence* of metabolising tissue. None of them replied, so I made my request public through the International Information Exchange, No 1, Scientific Memo,# 298 on the 14th of January, 1965. There was still no response.

I am now putting these findings on the Internet, to elucidate if the above findings can be repeated, and if they have any relevance to those described by the pioneers of this important field, Belousov, Zhabotinsky,Shnoll, Epstein, Pojman and others. I note that Amat (2009) has published a book on this subject, but I have not yet been able to obtain a sight of it. I wish to thank my daughter, Ms Rachel Hillman, for assistance with computer, and Mr. Stephen Ling-Winston and Mrs. Ellen Ling-Winston for their help with the Internet.

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