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MEASUREMENT OF THE BLOOD VOLUME BY MEANS OF BLOOD CORPUSCLES LABELLED WITH P^{32}

BY

HANS H. BOHR

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Introduction.

As described in several papers by HEVESY[1, 2, 3], it is possible to use radioactive phosphorus (P^{32}) in the determination of the total amount of circulating erythrocytes in the organism by labelling the red blood corpuscles *in vitro*.

The aim of the present work is to point out some circumstances that might contribute to a more rational view both on the labelling of the red blood corpuscles and on the conditions for the preservation of constant activity in the blood corpuscles, thereby considering especially the application of labelled blood corpuscles in the determination of the blood volume.

Earlier investigations by means of this method, primarily by HEVESY and collaborators, have shown that determinations on the same patient can be made with an uncertainty of 5—10 per cent and that the results are consistent with blood volume determination by means of CO and T-1824 on normal persons. NYLIN and coworkers have carried out numerous experiments[4, 5, 6] with the P^{32} method. Thus, NYLIN has determined the changes in the circulating of blood before and after pulmonectomy and before and after application of a tourniquet to an extremity, whereby the blood volume in the lungs and in a limb, respectively, could be measured. Owing to special conditions to which we shall return later, this author finds that in the blood corpuscles the activity remains practically constant for 1—2 hours, and utilizes the experience to examine the changes in the blood volume during this period, e. g. in patients with heart diseases. KELLY et. al.[7] have arrived at results in accordance with those of NYLIN. Recently REEVE and VEAL[8], when comparing the results from the P^{32} method with those gained by means of the T-1824 method, were able to show consistency. However, these authors propose

a technique somewhat different from that used by HEVESY and by NYLIN. After activation of the blood they remove the plasma previous to injecting the sample into the vein. This is done under the consideration that the labelled blood corpuscles alone are indispensable for the determination of the total amount of erythrocytes and that, therefore, the presence of the plasma in activity determinations is superfluous and even harmful. In their investigations REEVE and VEAL find that the cleaned erythrocytes lose some of their activity both *in vitro* and still more *in vivo*, the activity decreasing with 5 and 9 per cent, respectively, within 60 minutes.

Technique.

In our experiments the measurement of the blood volume was carried out in the following way. A sample of blood from a patient is shaken in a bottle containing radioactive phosphorus. Shaking should be performed very cautiously so that the blood corpuscles are kept under as physiological conditions as possible; it has to be done in a thermostat in order that the procedure may pass fairly quickly, cf. HEVESY and HAHN[1]. After two hours' shaking the amount of inactive phosphorus exchanged with active phosphorus is so large that the activity in the corpuscles is about the same as that in the plasma (per cc.). When the activated blood corpuscles are injected into the patients vein and the activity produced in the blood after its complete mixture with the injected sample is measured, it will be possible from the dilution found to calculate the total amount of blood corpuscles according to the following equation

$$X = p \cdot \frac{A}{a}, \qquad (1)$$

where X is the amount of blood corpuscles present, p is the injected amount of blood corpuscles, and A and a are the activities of the blood corpuscles in the injected sample and in the patients blood after the mixing, respectively.

The measurement of the radioactivity in the samples was originally carried out as a determination of the activity per gram of phosphorus. The blood corpuscles were destroyed by

boiling in nitric acid and sulphuric acid, a certain quantity of phosphate was added and, finally, the phosphate was precipitated as ammonium magnesium phosphate with Fiske's solution. Already in 1945 NYLIN suggested to measure the activity on dried and pulverized blood corpuscles. Recently, K. ZERAHN[9] has further simplified the method by measuring directly on the centrifuged blood corpuscles after having placed them in cuvettes. According to ZERAHN, these cuvettes are made of a steel ring with bottom and lid either of cover glass, 0.1—0.2 mm. thick, or of thin aluminium foil. Having acquired some experience one finds no special difficulties in filling and cleaning the cuvettes, which is done through one or two holes in the steel ring. The radioactivity is measured with an apparatus as described by AMBROSEN, MADSEN, OTTESEN, and ZERAHN[10] by means of an automatic arrangement similar to the one described in that paper.

Discussion.

As stated by HEVESY et al. in accordance with HALPERN, EISENMANN et al.[11] and TAYLOR et al.[12], the activation of the blood corpuscles is due to an exchange between the inorganic phosphate of the blood corpuscles and that of the plasma, and by an incorporation of inorganic corpuscle phosphate into organic acid soluble phosphorus compounds. According to HEVESY and ATEN[13], both these processes together are, at least in the beginning, directly proportional to the difference between the specific activity $\left(\frac{P^{32}}{P^{31}}\right)$ of the plasma and the specific activity of the blood corpuscles, depending on a constant denoted as the penetration factor a.

It is evident, however, that the penetration of P^{32} into the blood corpuscles must be a reversible process, radioactive phosphorus from the plasma being exchanged with inactive phosphorus of the blood corpuscles, and vice versa.

This can be expressed more clearly by the following equation:

$$dx = -b \cdot x \cdot dt + a \cdot (1 - x) \cdot dt, \qquad (2)$$

where it is merely indicated that the increase in activity of the blood corpuscles, dx, is the result of P^{32} entering the blood

corpuscles, $a \cdot (1 - x)$, and of P^{32} leaving the blood corpuscles, $b \cdot x$, a and b being coefficients for the processes involved and 1 being the total concentration of P^{32} in the blood.

By integration of equation (2) we obtain

$$X = \frac{a}{a+b} - C \cdot e^{-(a+b)t}, \tag{3}$$

where C is a constant;

if $X = 0$, when $t = 0$, then $C = \frac{a}{a+b}$, and the equation (3) becomes

$$X = \frac{a}{a+b} \cdot (1 - e^{-(a+b)t}); \tag{4}$$

if $X = 1$ when $t = 0$, then $C = \frac{-b}{a+b}$. Subsequently, we obtain

$$X = \frac{1}{a+b} \cdot (a + b \cdot e^{-(a+b)t}). \tag{5}$$

Equations (4) and (5) are expressions of the activation process in blood, starting with the total activity present in the plasma and in the red blood corpuscles, respectively. The coefficients a and b can be explained in the following way. When X is very small, practically no radioactive phosphorus will leave the corpuscles. From equation (2) one gets

$$\frac{dx}{dt} = a \quad \text{for} \quad X = 0;$$

a therefore expresses the slope at the beginning of the curve for the equation (4). Similarly, we get

$$\frac{dx}{dt} = -b \quad \text{for} \quad X = 1,$$

which means that $-b$ expresses the slope at the beginning of a curve representing the diffusion of P^{32} from the corpuscles into the plasma (equation (5)). If, in equations (4) and (5), $t = \infty$, we get

$$X = \frac{a}{a+b}$$

which means that both curves approach this value. For the special problem here discussed, a is much larger than b since the amount of exchangeable phosphorus is much larger in the corpuscles than in the plasma. Fig. 1 gives three examples of curves representing three different sets of values for a and b.

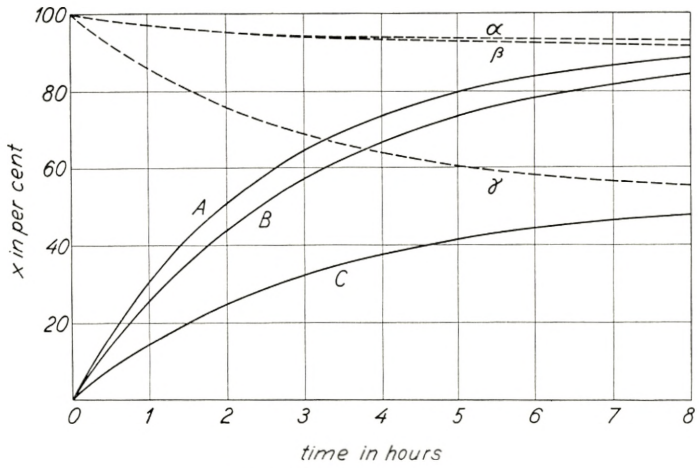

Fig. 1. Theoretical curves.

The lower curves illustrate the equation $x = \frac{a}{a+b}(1 - e^{-(a+b)t})$.

The upper curves illustrate the equation $x = \frac{1}{a+b}(a + b\, e^{-(a+b)t})$.

Curve A and α corresponding to a = 0.37, b = 0.03.
Curve B and β corresponding to a = 0.30, b = 0.03.
Curve C and γ corresponding to a = 0.17, b = 0.16.

From these considerations it appears that the activation process is independent of the absolute activity of the blood corpuscles and the plasma, but dependent only on the relation between the activity of the blood corpuscles and that of the plasma. This means that the course of the process will not be changed when fresh blood from the same person is added, in which case the blood corpuscles and the plasma would be equally diluted. According to the assumptions underlying the calculations above it makes no difference whether the activity is distributed uniformly in the blood corpuscles or some corpuscles have more and some less activity.

If, however, plasma or physiological NaCl solution is added

only, the relation between the activity of the plasma and that of the blood corpuscles will be different and, consequently, the course of the process will be changed.

Experimental.

In order to elucidate whether this theory is in accordance with actual conditions, experiments *in vitro* were carried out. Newly drawn blood kept in paraffinated bottles to which heparin had been added was used. After addition of P^{32} of negligible weight the bottles were carefully shaken in the thermostat at 37° C. At convenient intervals samples were drawn and sharply centrifuged, and the activity of the blood corpuscles and the plasma was determined. Cuvettes of the type described previously were used in these experiments.

Fig. 2 gives an illustration of the increase in activity of the blood corpuscles. By measuring the activity in percentage of the total activity of the blood, the hematocrit value is taken into account and, therefore, more constant conditions are obtained for the individual curves, which still, however, show some differences. The average curve of 12 such experiments is shown and compared with the theoretical curve for $a = 0.37$ and $b = 0.03$. Individual values for four different experimenting curves are given as points of various kinds (Fig. 2 a), the experimenting error being about $\pm 2\ \%$. The value of b is obtained by drawing curves on the diffusion of P^{32} from the blood corpuscles. First, the blood corpuscles are activated; then the blood is sharply centrifuged, the plasma removed with a pipette, and the blood corpuscles are resuspended in a cooled physiological NaCl solution. This process is repeated and, after a second centrifugation and removal of the NaCl solution, inactive plasma from the patient from whom the blood sample was drawn is added. The blood is shaken in the usual way in the thermostat, samples being removed for the determination of the activity of the plasma and the corpuscles. The average for b as obtained from two experiments is 0.03 (Fig. 2 b). Hilde Levi[14] in her experiments has obtained curves only slightly deviating from these.

A comparison between the curves in Fig. 2 shows that the

agreement between the theoretical and the experimental curves is satisfactory. However, the experimental curve does not rise to higher values than 0.86, while the theoretical maximum for a = 0.37 and b = 0.03 is 0.93, according to the above-mentioned expression $\frac{a+b}{a}$. This can be accounted for in the following way. When examining freshly drawn blood, the amount of acid

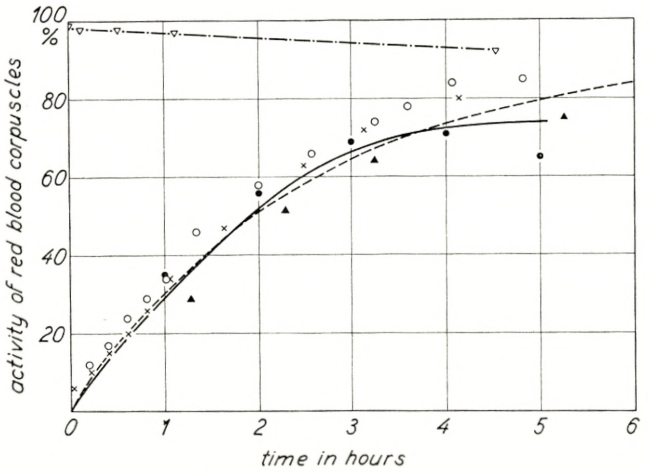

Fig. 2. Experimenting curves.
The lower curves representing Fig. 2 a.
The upper curve representing Fig. 2 b.
The dotted line gives the theoretical curve for a = 0.37, and b = 0.03.
The full line gives the average values for 12 experiments.
The different points showing individual values of 4 experiments.

soluble phosphorus (which includes that amount of phosphorus which is easily exchangeable within the time of observation) in the plasma and the blood corpuscles is found to be about 38 and 3 mg %, respectively. These figures are in good agreement with the values found for a and b. But in the course of 4 hours' incubation at 37° C. some of the phosphorus compounds of the blood corpuscles are destroyed with the result that the amount of inorganic phosphate of the plasma is increased to about 5 mg %,. This phenomenon diminishes the relation $\frac{a}{b}$, and consequently the curve does no more rise to the same level.

Thus, it seems probable that the blood *in vitro* reacts as calculated from the theoretical considerations. Indeed, if some

activated blood is mixed with a larger amount of inactive blood and the activity of the blood corpuscles is investigated, it is found that the changes of the activity are not influenced by this dilution which does not change the relative activities of the plasma and the blood corpuscles.

Different results are obtained when these proportions are changed, for instance by diluting the plasma with physiological NaCl solution, as shown in Fig. 3. Curve 2 makes it clear that if so much NaCl solution is added that the activity of the plasma decreases to $\frac{1}{10}$ of the activity of the blood corpuscles, the activity of the blood corpuscles remains constant. If only half as much NaCl solution is added, the activity of the blood corpuscles is hardly changed either. If we dilute with still less NaCl solution, the activity of the blood corpuscles continues to increase, though more slowly than before. If we dilute so highly that the activity of the plasma decreases to less than $\frac{1}{20}$ of the activity of the blood corpuscles, the corpuscles will lose some activity until a balance is restored (Fig. 3, 3). These experimental results are in agreement with the theoretical considerations outlined above.

When attempting to utilize these experiences at the application of the radioactive blood corpuscles in the determination of the blood volume *in vivo* we find that the blood after activation for two hours is not yet in balance, i. e. the activity of the blood corpuscles is still increasing. On injection in the vein, the radioactive blood will be diluted with inactive blood of the same composition, whereby the activation process will not be affected. However, a special circumstance has to be taken into consideration, viz. the activity of the plasma which is present mainly as inorganic phosphate will, after the injection, distribute *not* only over the plasma, but also over the extracellular fluid and the activity will decrease correspondingly. If we follow the activity of the blood corpuscles and of the plasma after injection of the activated blood, we find that the activity of the blood corpuscles remains constant during the first hour (cf. also (5)), while the activity of the plasma even after completion of the mixing continues to decrease very rapidly during the first 10 minutes; from then on the activity still decreases, though at

a slower rate. The steep decrease in the plasma activity during the first 10 minutes, which is shown in Fig. 7, is an expression of the exchange of inorganic phosphate between plasma and extracellular fluid (cf. HAHN and HEVESY[15]). Thus the activity of the plasma in the course of a very short time will decrease to about one fifth of the activity of the blood corpuscles, and according to results shown in Fig. 2 and 3 the activity of the

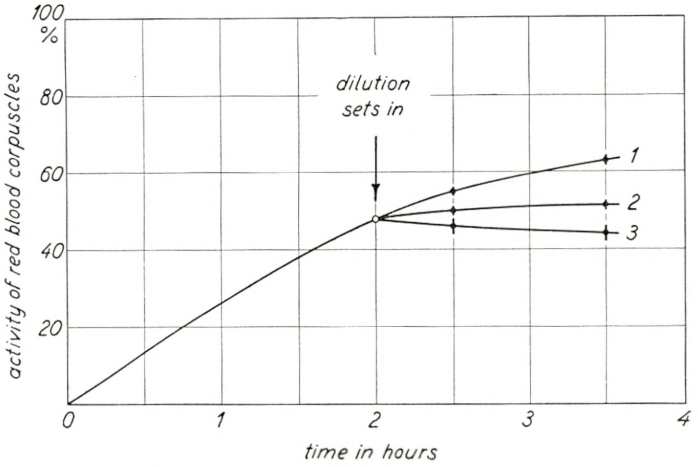

Fig. 3. Curves showing the effect of diluting.
1) 5 cc. labelled blood to 30 cc. inactive blood. (Experimenting error ± 2 %)
2) 20 cc. labelled blood to 50 cc. phys. saline. (— — ± 2 %)
3) 20 cc. labelled blood to 250 cc. — — (— — ± 5 %)
Hematocrit value 44 %.

blood corpuscles will consequently remain almost unchanged. The activity of the plasma, however, will, as mentioned above, continue to decrease as a result of the interchange of the plasma phosphate with intracellular phosphate and through excretion.

If the activity of the plasma decreases much below one tenth of the activity of the corpuscles, then the latter will diminish somewhat. As already mentioned, REEVE and VEAL proposed to remove the plasma from the blood corpuscles prior to the injection. Following this procedure, as was to be expected, a loss of activity is observed in one hour.

As mentioned before, it can be shown that blood kept in a thermostat undergoes a change. If we follow the experimental curve in Fig. 2 for 8—10 hours, we see that it deviates still more from the theoretical curve. Fig. 4 shows how the curve now

begins to fall, reaching quite a new balance which remains constant for more than 48 hours. This fall precedes any hemolysis, since in most cases no red-colouring of the plasma can be observed until after 10 hours' shaking, when it begins very faintly and then increases steadily. Neither does one see any changes in the hematocrit value nor on the red blood picture. The phenomenon must be regarded as an expression of changes

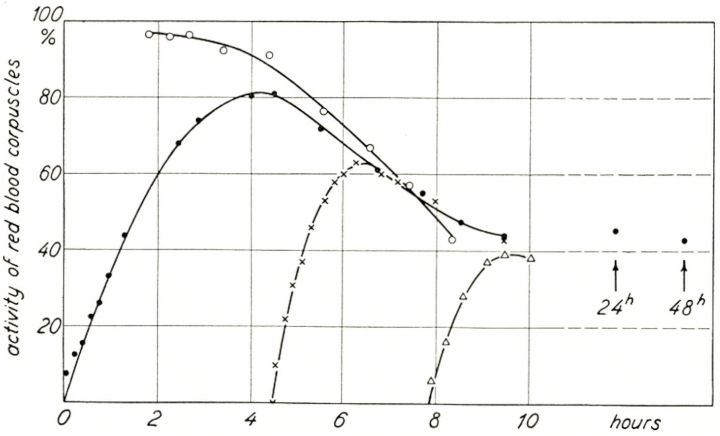

Fig. 4. Curves showing labelling of the red blood corpuscles.
● fresh blood.
× blood stored at 37° for 270 min.
Δ blood stored at 37° for 470 min.
○ blood activated for 37° for 90 min., replacing the plasma with inactive plasma.

inside the blood corpuscles, in the first place probably with regard to the sensitive enzymatic system regulating the phosphorylation processes. This is also indicated by an accompanying steep increase in inorganic phosphate of the plasma, as shown in Table 1.

A closer examination of these conditions shows that the decrease in the activity of the blood corpuscles is directly dependent on the time during which the blood is kept at 37° C. If the blood corpuscles were placed in a thermostat for 4 hours before addition of P^{32}, and then shaken, the result is seen on Fig. 4, which also shows the course of the activation when the blood before activation was kept in a thermostat for 8 hours. A curve like that does not reach beyond the values corresponding to the secondary balance.

The shape of the activation curve of the blood corpuscles treated with P^{32} thus indicates a way of estimating the extent to which the blood is changed during storage.

It might, however, be that such a change was produced as a consequence of the fact that the blood consumed some substance important for the processes involved. Here, one might

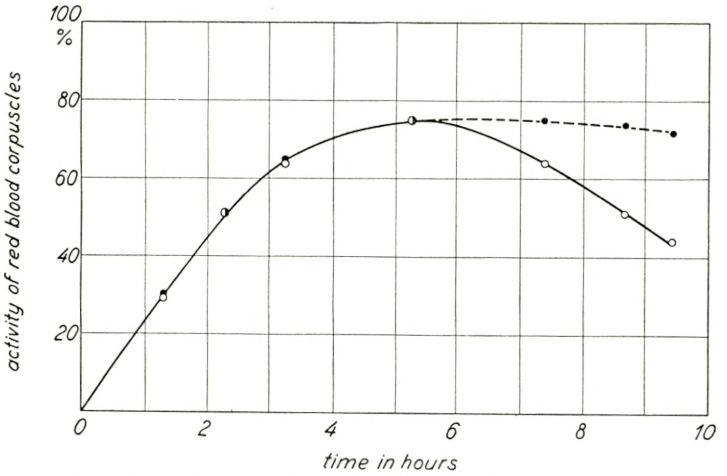

Fig. 5. Activity curves showing the effect of glucose.
Dotted line representing blood to which glucose was added during incubation.
Full line representing blood to which no glucose was added.

think of oxygen, for instance. However, as the bottles containing the blood are made so as to secure an ample supply of oxygen, no analysis of the air was performed. Another substance, the presence of which is of equal importance in this respect, is glucose. As is well known, the glucose content of the blood decreases rather rapidly during incubation at 37°. This is shown in Table 1 where glucose content of the blood is seen to fall from about 80 % to about 20 % in the course of a few hours. The effect of an addition of glucose to the blood is also shown by Table 1, which demonstrates that the presence of a high concentration of glucose in the blood reduces the loss of P^{32} by the corpuscles. But even if the loss of glucose is replaced, the corpuscle activity still decreases as shown in Fig. 5 indicating that some change has taken place in the blood.

If blood is stored at room temperature for 24 hours we obtain

Table 1.

With Glucose			Without Glucose		
Min.	P mg %	Glucose mg %	Min.	P mg %	Glucose mg %
0	2.9	70	0	2.9	70
77	..	29	77	..	54
137	3.1	25	137	3.3	156
316	4.7	..	316	4.4	..
441	8.7	..	441	6.0	..
515	14.7	..	515	8.2	..
563	..	25	563	..	150

an activation curve similar to that of blood kept at 37° C for 8 hours; if however, glucose is added in suitable amounts, the activation curve is found to be identical to that obtained for freshly drawn blood, demonstrating that no irreversible change takes place within 24 hours at room temperature.

It is not surprising that glucose plays an important part in

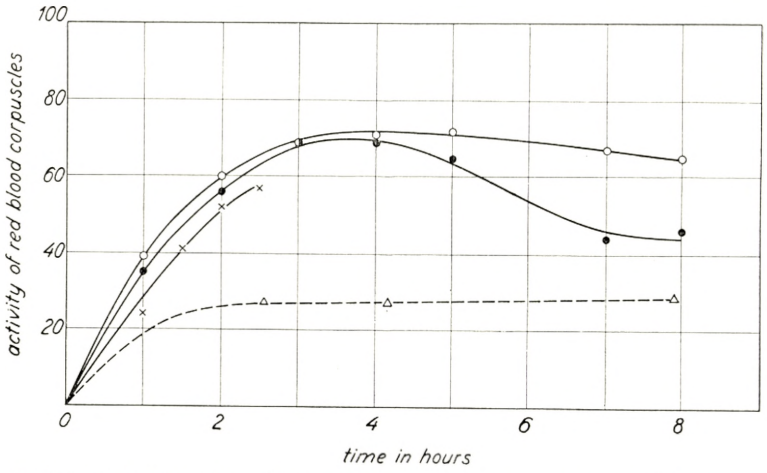

Fig. 6. Curves showing the effect of different agents on the labelling of blood.
● blood with heparin.
○ blood with acid citrate-glucose solution.
× blood with 3 % citrate.
Δ blood with 2 ‰ fluoride.

the activation of the corpuscles as glycolytic processes are involved in the incorporation of P^{32} into the organic acid soluble phosphorous compounds. In an experiment in which fluoride

was added to the blood the rate of intrusion of P^{32} into the blood corpuscles was found to be much reduced. This is shown in Fig. 6, which also demonstrates the behaviour of blood containing 3 % citrate or acid citrate-glucose solution, respectively. None of these substances seem to influence the interchange mechanism of the corpuscles.

Conclusion.

From the results above, the following conclusions may be drawn. To obtain a constant activity of the blood corpuscles, the activity of the blood corpuscles must be about 10 times as large as the activity of the plasma (per cc.). This equilibrium can be temporarily obtained *in vivo* by injecting blood of almost equal activity of the blood corpuscles and the plasma, since the activity of the plasma decreases after an exchange with the extracellular fluid. According to Fig. 2, an activation of about 2 hours at 37° C. is required to obtain this ratio between the activities in the blood. It appears, however, that blood *in vitro* kept at 37° C. gradually undergoes a change, so that a shorter time of activation is prefer-

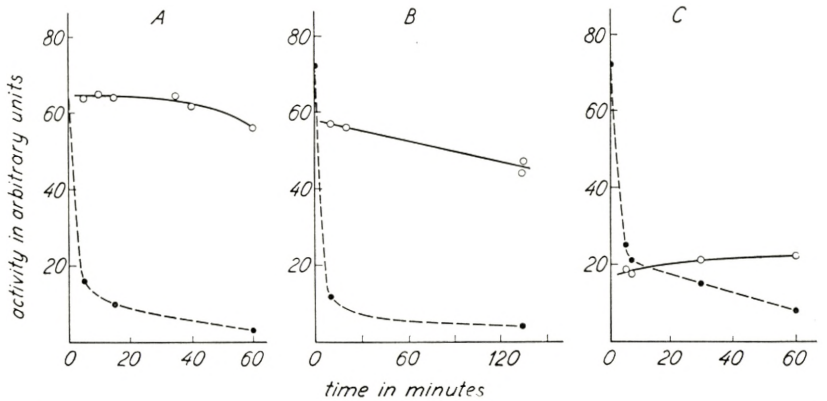

Fig. 7. Curves showing the activity of the blood corpuscles (full line) and the plasma (dotted line) following injecting *in vivo*.
A following injection of blood labelled for 120 min.
B following injection of blood labelled for 75 min.
C following injection of blood labelled for 40 min.

able. By activating for one hour, only, the activity of the plasma appears to be relatively too high in the beginning, but in the course of some minutes it decreases sufficiently not to cause an

increase in activity of the blood corpuscles, due to the continual cellular uptake and excretion of phosphate. If, however, the blood is activated for half an hour only, the activity of the plasma will cause an increase in the activity of the blood corpuscles (Fig. 7). Still, it is possible to reduce the time of activation considerably by removing some of the activity of the plasma. This can be done by centrifuging the blood, as suggested by REEVE and VEAL. But while these authors wash the corpuscles in cooled physiological saltwater to remove all the plasma, thereby easily damaging the red blood corpuscles, the above considerations seem to indicate that it is more reasonable to remove only the greater part of the plasma after very delicate centrifugation. Which method is to be preferred—either activating for one hour or activating for about 20 minutes and centrifuging of the blood — will depend upon the results to be obtained *in vivo* by each method.

Summary.

1. Theoretical considerations in connection with the determination of the blood volume by means of erythrocytes labelled with P^{32} are put forward, and their correctness tested by measurement of the rate of interchange between plasma and blood corpuscles in experiments carried out *in vitro*.

The results obtained permit a satisfactory explanation of the data obtained by various experimenters.

2. The rate of loss of P^{32} by the activated corpuscles was found to increase markedly in blood incubated at 37° for more than 5 hours. Addition of glucose reduces the rate of loss of P^{32} by the corpuscles.

3. The most favourable way of obtaining labelled corpuscles is discussed.

The present investigations were carried out at the University Surgical Clinic C., Copenhagen, and at the Institute for Theoretical Physics, University of Copenhagen. I wish to thank the heads of these institutions, Professor E. DAHL-IVERSEN and Professor NIELS BOHR for their continuous interest and encouragement. I am especially indebted to Professor G. HEVESY for most valuable guidance and advice throughout the work.

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