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The Stability of PGM and AK Isoenzymes in Human Tissues

Many blood group systems are known and are in regular use in blood transfusion laboratories. Such laboratories deal with fresh samples of blood in good condition. The forensic laboratory, on the other hand, mostly must work with dried blood in the form of stains on many different substrates and in all types of condition, and the only blood grouping tests suitable for forensic use are those in which the group factors are stable to drying. In this context it is useful to know how stable the various blood group factors are and to determine whether these factors change with age, for this could lead to confusion in typing the material.

Several enzymes present in the red blood cell exist in various genetically determined molecular forms known as isoenzymes. These forms are readily identified using electrophoretic techniques. Two of the enzyme systems in use in many forensic laboratories are phosphoglucomutase (PGM) (Spencer et al [*1*]) and adenylate kinase (AK) (Fildes and Harris [*2*]). Previous work by Rothwell [*3*] has shown that these isoenzymes can be detected in bloodstains up to several months old, and in blood samples maintained at −20°C for periods well in excess of a year. This work also indicated that in both liquid samples of blood and bloodstains the PGM enzyme system is less stable than the AK enzyme system, so that the probability of being able to type a sample decreases more rapidly with time for the PGM than for the AK system.

Neither PGM nor AK enzymes are confined exclusively to erythrocytes; in particular, phosphoglucomutase plays an important role in glycogenesis and glycolysis in both muscle and liver, while adenylate kinase (or "myokinase") aids in the production of the adenosine tri-phosphate necessary for muscle contraction.

In view of the fact that requests are sometimes made to the forensic laboratory to perform tests to ascertain the blood group of human tissue fragments, and as recent work by Pereira [*4*] has indicated that no reliability can be placed on the determination of the ABO blood group substances in decomposing human tissues, it was decided to investigate the stability of PGM and AK in tissues other than blood. It was hoped that such work would indicate the activity of enzyme present in the tissue, whether the enzyme existed in isoenzyme forms correlated with those present in blood, the stability of the enzymes, and, most important, whether the deterioration of enzyme in the tissue would lead to samples being wrongly typed.

The following tissues were examined in this study: (1) adipose tissue, (2) brain, (3) muscle, (4) liver, and (5) blood.

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The first three were selected because tissues from these sources are sometimes found during forensic science examinations, for instance, on motor vehicles involved in road traffic accidents or on weapons used in assaults. Liver was included in the study as it was considered likely that it would contain high concentrations of enzymes, especially PGM. The enzyme types found in the tissues were compared with the types found in blood samples taken from the same individuals.

To examine the relative stabilities of the enzymes, samples of tissue were stored at room temperature, at 4°C, and at −20°C, and the enzymes were typed after varying periods of storage under these conditions.

Methods

Samples of tissue were removed at postmortem examinations from individuals of a range of ages who had died from a variety of causes. Approximately 100-g samples of each tissue were taken, together with about 10 ml of blood. The materials were immediately transported to the laboratory.

Preparation of Tissue Samples

The tissue samples were washed to remove any adhering blood and then divided into roughly equal portions, each weighing about 1.5 g. The small portions were rewashed; after this stage the muscle, adipose tissue, and brain all contained negligible quantities of blood, but it was not found to be possible to remove completely the blood from the liver samples. Each small portion of tissue (about fifty samples in total) was placed in a small screw-capped plastic tube, 4–5 drops of normal saline were added, and the tissue was macerated in the liquid, using a needle. Finally, the tubes were capped for storage, half of the samples of each tissue being kept at room temperature, with the other half of the samples maintained at 4°C. A sample of tissue was used for one enzyme determination and the tube was then discarded.

The tissues remaining from the original samples were then placed in small plastic tubs and stored at −20°C.

Preparation of Blood Samples

Ten-millilitre samples of blood taken at postmortem were centrifuged to separate the cells and the serum, which was discarded. The packed red cells, after washing three times with saline solution, were lysed by adding a few drops of distilled water. Each blood lysate was divided into half and placed in small screw-capped plastic tubes for storage. One half of each sample was maintained at 4°C while the other half was kept at room temperature. Previous work by Rothwell [3] has shown the effects of storage at −20°C on the PGM and AK enzymes of blood.

Detection of the Isoenzymes

The isoenzymes of PGM and AK were detected by thin-layer starch gel electrophoresis (Wraxall and Culliford [5]), using the methods of Culliford [6] for PGM determination, and Culliford and Wraxall [7] for AK determination. The starch gels were prepared using hydrolyzed starch (Connaught Medical Research Laboratories, Toronto, Canada).

Plain white cotton sheeting was boiled for one hour in distilled water and allowed to dry.

Single threads about 1 cm long were teased from the sheeting, and dipped either into one of the lysed blood samples or into one of the tubes of macerated tissue. The threads

were then inserted into the starch gel. Gels were subjected to electrophoresis for 17 h at 4°C, using a potential gradient of 5 V/cm. The isoenzyme bands were visualized on the gel as blue formazan complexes, which formed during incubation at 37°C in an agar overlay containing a substrate-reaction mixture for the particular enzyme being tested. Preliminary tests indicated that tissues could contain very high levels of PGM and AK enzymes, and therefore the arbitrary scale used in previous studies for recording the intensity of coloration of the enzyme bands was modified as follows.

Characterization of PGM and AK types not possible	0—no visible enzyme activity 1—some faint diffuse blue coloration, not in discrete bands
Characterization of PGM and AK types possible	2—discrete bands of moderate intensity 3—discrete intense enzyme bands, developing fully after 20–30 min of incubation 4—very intense bands, visible within 2–5 min of the overlay being poured onto the starch gel

A score of 3 units would normally be expected from a fresh blood lysate. As the experiments were intended to ascertain whether tissue samples could be reliably typed, absolute measurements of enzyme activity were not considered relevant.

The quality of the electrophoretic separation was controlled by including on each gel a thread bearing blood of known type, either PGM2-1 or AK2-1, depending on the enzyme system to be tested. In addition, to ensure that the reaction mixture in the overlay was functioning correctly, threads moistened with dilute solutions of either rabbit muscle PGM (C. F. Boehringer and Soehne, GmbH, Mannheim) or rabbit muscle myokinase (Boehringer) were inserted into the agar overlays prior to incubation.

Tissue samples were stored for 80 days, and were regularly tested for enzyme activity during this period of storage.

The enzymes in muscle were found to be still detectable after long periods of storage, and a number of samples were retained and tested after 160 days.

The tissue samples used included representatives of all the common types in the PGM_1 system (Types 1, 2-1, and 2) and in the AK system (Types 1 and 2-1).

Results

For ease of comparison of the results, the data obtained from the various times of storage of the tissues at 4°C and at room temperature have been collected together into five periods of 0–10, 11–20, 21–40, 41–60, and 61–80 days of storage postmortem.

As the enzymes were expected to be more stable in deepfrozen samples than in thos kept at higher temperatures, the tissues stored at −20°C were examined less frequentl tests being performed after 30 and 60 days of storage.

PGM Enzyme Activity

The activity of PGM enzyme in postmortem tissues is shown in Fig. 1. Each block on the histogram represents the mean of all the scores obtained from the individual tests performed during a particular period of storage; except where indicated, each mean score is the average of at least ten experimental results. On the arbitrary scale employed the enzyme group can be determined only if the activity score is 2 units or higher. All of the

tissues could initially be readily typed in the PGM system. As might have been expected, the activity decreased most rapidly in the samples stored at ambient temperature and least rapidly in the deepfrozen material. The PGM enzyme in muscle appeared to be particularly stable, and readable results were obtained from muscle samples stored at 4°C for up to 160 days.

Table 1 expresses these results in a different form, indicating what proportion of the tissues tested could be successfully typed in the PGM system.

The tissues stored at room temperature are probably the most relevant to forensic work; most of these samples could be typed during their first ten days of storage. Nine out of ten blood samples could be typed after storage for periods up to 20 days. It should be noted that after storage of more than ten days the activity of the "a" isoenzyme band became relatively weaker than that of the other PGM_1 locus bands in some of the samples stored at room temperature. This effect was particularly noticeable with muscle samples. Complete loss of this "a" band was noted in a few of the samples.

Successful typings were performed on more than half of the brain and adipose tissue samples kept for up to 60 days, and of the liver and muscle samples kept for up to 80 days at 40°C. All of the deepfrozen samples were successfully typed.

AK Enzyme Activity

The activity of AK enzyme in postmortem tissues is shown in Fig. 2, in which are shown the mean scores obtained after varying periods of storage. As before, each score represents the average of at least ten experimental results.

High levels of this enzyme were found in blood, muscle, and to some extent in liver samples. The enzyme activity in some adipose and brain tissue samples was so low that they could not be typed, even at the start of the storage period. As with the PGM enzyme, the loss of activity with time was greatest for samples stored at room temperature and least for those kept at −20°C.

The proportion of tissues successfully typed after various storage intervals is shown in Table 2. Most of the blood, brain, liver, and muscle samples stored at room temperature could be typed for up to ten days following postmortem removal, but only a third of the adipose tissue samples were typed during this period. Only blood retained a high AK activity for a significantly longer period. The enzyme was found to be more stable at 4°C, and most of the samples stored at this temperature could be typed for up to 20 days; many of the blood and muscle samples retained their activity for much longer. Of the tissues which were deepfrozen immediately upon their postmortem removal, only adipose tissue lost so much enzyme activity that most of the samples could not be typed. With some samples it was observed that after storage the "2" band of the AK2-1 variant was often less intense than the other bands.

No incorrect PGM or AK types were assigned to any of the samples tested in this series of experiments. Provided that discrete enzyme bands were produced in the starch gel following incubation with the reaction mixture, the isoenzyme type was immediately obvious; no attempt could be made to type those samples which did not produce a clear band pattern.

Discussion

These experiments indicated that both PGM and AK isoenzyme variants may be successfully detected in tissues other than blood; as the tissues decomposed the enzyme activities decreased, although both enzymes could be detected in muscle and liver tissues which, to judge by their odor and appearance, were in an advanced state of decomposition.

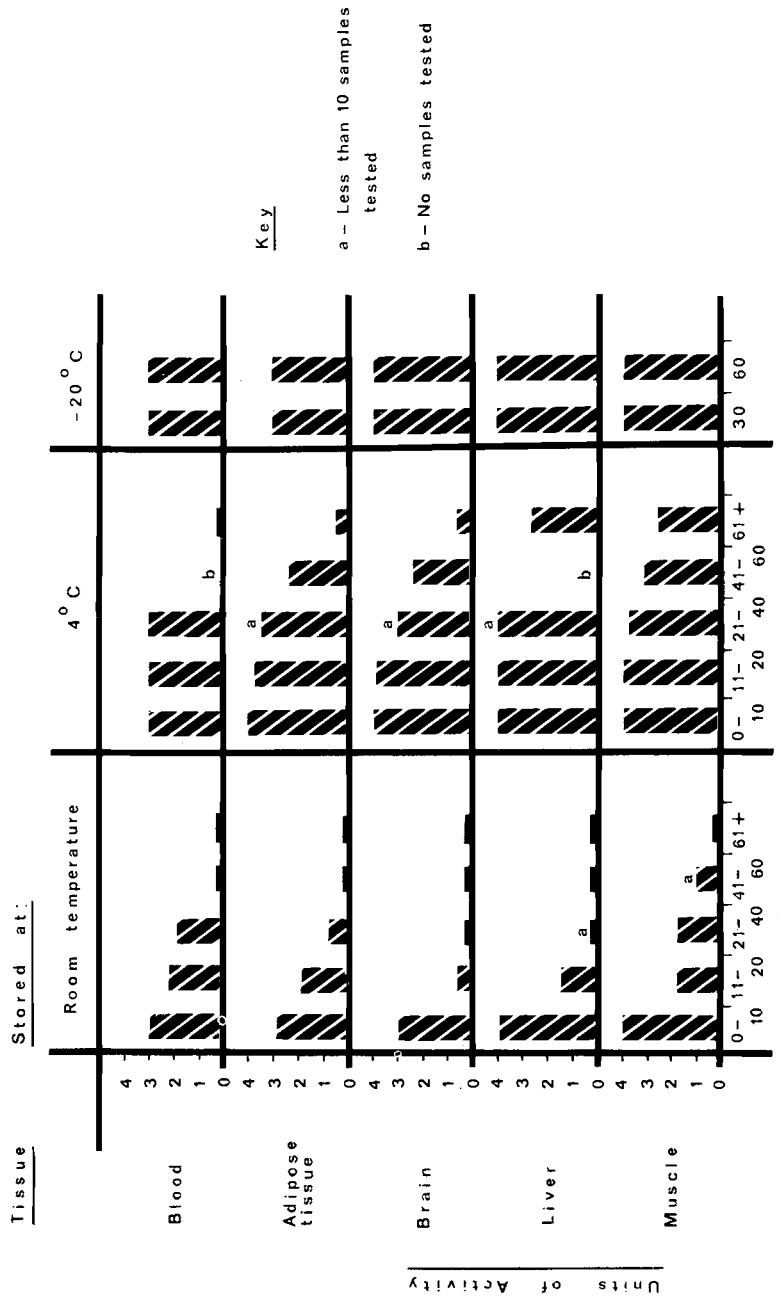

FIG. 1—*Stability of PGM enzyme in human tissues.*

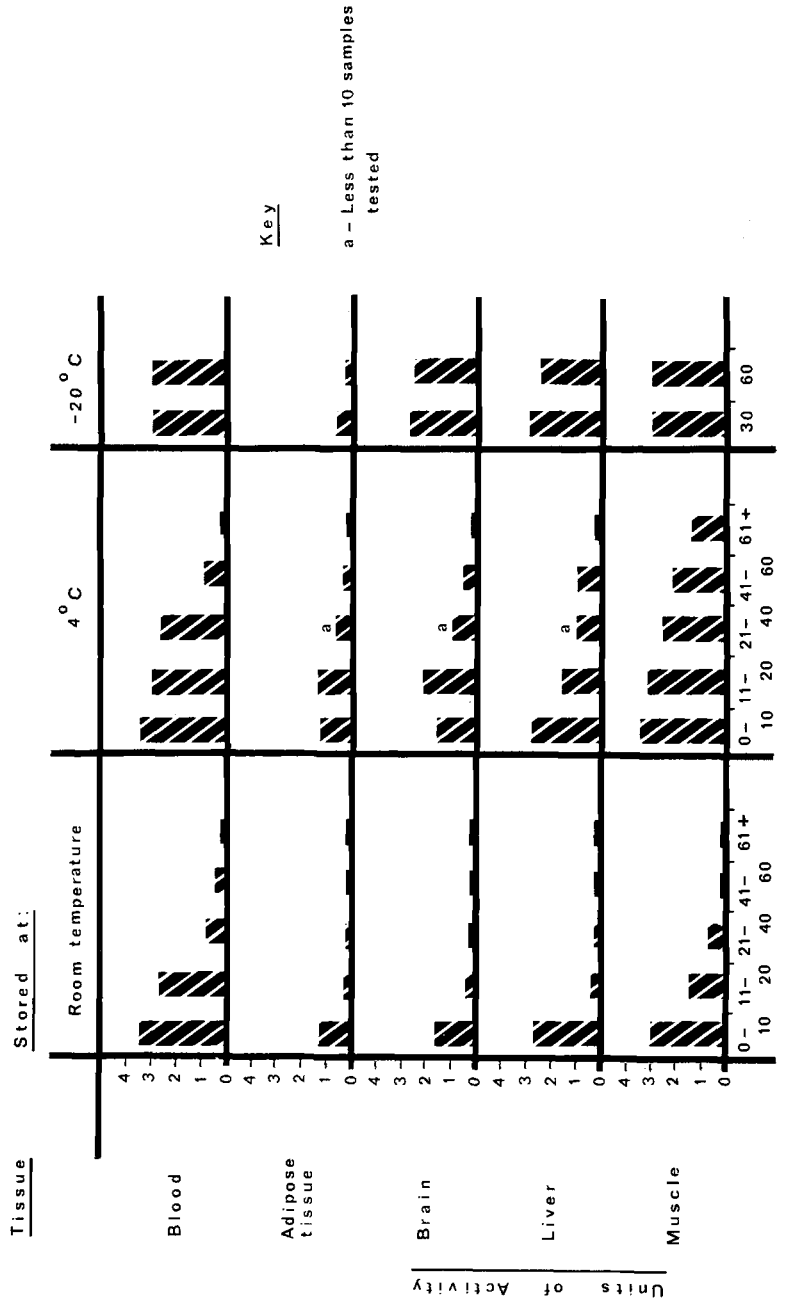

FIG. 2—*Stability of AK enzyme in human tissues.*

TABLE 1—*Proportion of tissues typed correctly after various storage times—PGM enzyme expressed as a percentage of the tissues examined.*

	Stored at Room Temperature for					Stored at 4°C for					Stored at −20°C for	
Tissue	0–10 days	11–20 days	21–40 days	41–60 days	61+ days	0–10 days	11–20 days	21–40 days	41–60 days	61+ days	30 days	60 days
Blood	100	90	40	0	0	100	100	80	X	0	100	100
Adipose	88	50	25	0	0	100	100	67	60	20	100	100
Brain	88	10	0	0	0	100	88	67	60	20	100	100
Liver	100	40	0	0	0	100	100	67	X	60	100	100
Muscle	100	45	53	0	0	100	100	94	80	80	100	100

X = No samples tested.

TABLE 2—*Proportion of tissues typed correctly after various storage times—AK enzyme expressed as a percentage of the tissues examined.*

	Stored at Room Temperature for					Stored at 4°C for					Stored at −20°C for	
Tissue	0–10 days	11–20 days	21–40 days	41–60 days	61+ days	0–10 days	11–20 days	21–40 days	41–60 days	61+ days	30 days	60 days
Blood	100	100	30	20	0	100	100	100	40	0	100	100
Adipose	33	0	0	0	0	29	55	0	0	0	100	80
Brain	50	0	0	0	0	43	78	33	20	0	20	0
Liver	83	0	0	0	0	100	55	0	0	0	100	80
Muscle	83	36	15	0	0	100	100	79	73	40	100	100

During the experiments on PGM determination on tissues which were stored at room temperature, it was noted that the activities of the various enzyme bands did not all decrease at the same rate. For example, in samples stored under these conditions for more than 10–12 days there was a tendency for the "a" band in Types PGM1 and PGM2-1 samples to be very weak or nonexistent. In such cases the banding can superficially resemble that of a Type PGM2, and it is therefore essential to be certain which of the other PGM_1 locus isoenzyme bands—b, c, and d—are also present in the sample under investigation. It occasionally happens that the "b" and "c" isoenzyme bands do not separate well on a particular PGM plate; under such conditions it would appear to be unwise to attempt to read the type of any tissue samples included on that plate. Culliford [*8*] notes the inverse relationship between the activities of the "a" and "c" isoenzyme bands with varying tissue age. A similar relationship was observed in this study, the "c" band frequently exhibiting enhanced activity in those samples in which the "a" band was less intense.

Although similar effects were noted with AK enzymes, in that the "2" band appeared to be less stable than the other enzyme bands, in no sample tested was the "2" band so much weaker than the other bands that an AK Type 2-1 was mistakenly identified as a Type 1.

Many of the tissue samples initially displayed much higher levels of PGM enzyme than are normally found in freshly lysed blood samples, and the enzyme persisted for a very long time when the tissues—particularly muscle and liver—were stored at 4°C or lower temperatures.

As might be expected, the enzymes deteriorated much more rapidly at room temperature. Although most of the tissue samples displayed some AK activity initially, only in muscle samples did this activity persist for more than a few days.

It is interesting to compare the results obtained from tests on blood samples kept at room temperature with the results previously obtained by Rothwell [*3*] on bloodstains. There was found to be at least an even chance of grouping stains in the PGM system after three months and in the AK system after five months, but only in those liquid blood samples less than 20 days old was there a more than even chance of successfully typing the enzymes.

These findings may reflect the greater potential for micro-organism growth in the liquid medium than on the dry stain; from this it may be inferred that the small fragments of dry tissue on weapons, etc, which are of interest in the forensic context should be typable for at least as long and probably longer than the samples examined in these experiments.

It was also found that, in bloodstains, AK was the more stable of the two enzymes. The present studies on liquid blood samples show a similar tendency. However, in the other tissues tested, the PGM was the more stable enzyme.

This series of experiments has shown that these two enzyme grouping systems can be of great value to the forensic scientist during the examination of other human tissues as well as blood. The results have already been applied to casework in this laboratory; in a fatal hit-and-run traffic accident a minute quantity of human adipose tissue was found adhering to the damaged area of the vehicle involved. The tissue fragment was much too small to be grouped using a serological technique. However, there was ample material for PGM typing, and the tissue was found to be Type PGM2, the same group as was present in the deceased's blood.

Summary

Isoenzymes of both PGM and AK have been detected in various human tissues. The enzyme variants were found to correspond to the types found in blood. Although many tissues initially give very strong reactions in both PGM and AK typing systems, the enzymes deteriorate fairly rapidly unless the samples are kept in a refrigerator. Muscle, in particular, contains very stable enzymes which can be detected after several months of storage at 4°C or in deepfreeze. Successful typings were obtained from material in an advanced state of decomposition.

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