was inhibited by lowering the surface temperature sufficiently. The duration of pressure rather than the intensity appeared to be the important factor.

2. Pressure on strips of skin isolated from the body produced no change in the state of dispersion of the melanophores contained therein. However, when pressure was applied to excised blocks of tissue consisting of skin and underlying muscle, concentration of the melanophores occurred just as if the tissue were still a part of the dead fish. This suggested that pressure brought about the transfer of some melanophore-concentrating substance from the muscle or subcutaneous tissues into the skin.

3. Strips of skin removed from the trout and replaced on the subcutaneous tissue showed no effect from applied pressure. The result was quite different, however, when a skin strip was laid on exposed muscle. Marked melanophore concentration and pallor took place within a minute or two. Extracts of the dead trout's muscle made by maceration in 0.9% NaCl solution were equally effective.

4. That pressure-induced pallor was not simply a post-mortem effect was shown by placing a rubber band around the live trout. Its removal after 45 min revealed a band of pallor encircling the fish's body.

5. Evidence that living muscle is capable of liberating a melanophore-concentrating substance was obtained by laying a strip of skin (from another trout) on the exposed muscle of a live trout with its head kept under water during the procedure. Concentration of the melanophores of the skin strip at points of contact with the muscle was just as rapid as when muscle of dead trout was employed.

Of the known substances in muscle that possess the power to produce concentration of melanophores—namely, potassium and calcium—the former exhibits considerably greater activity in this respect (*1*) and is very much more abundant than calcium. Furthermore, since potassium constitutes by far the predominant base of muscle, it is unlikely that any other ion (e. g., magnesium) could be responsible for this effect. Determinations were made of the potassium content of trout muscle (freed from fat) and of extracts (equal parts by weight of muscle and 0.9% NaCl solution, ground in a mortar) in order to ascertain whether the concentrations of potassium present were sufficient to produce the degree of melanophore contraction described. Trout muscle was found to contain 129 mEq potassium/kg muscle, which is a relatively high figure for vertebrate muscle. The muscle extract contained 66 mEq/kg fluid,[2] which in terms of KCl would equal 0.49% solution. It was found in the previous study that 0.8% NaCl was required to neutralize the melanophore-contracting effect of 0.1% KCl. Actual tests with 0.5% KCl showed that even 2% NaCl, which by itself produces a marked dispersion of the melanophores, when added to the KCl solution did not prevent the contracting effect of the potassium. Another pertinent observation of the earlier study was that the potassium ion is able to penetrate rapidly the subcutaneous surface of the isolated skin strip.

Although these findings made it evident that release of potassium from the trout's muscle could account for the above-described effects on the melanophores, they did not exclude the possibility that the contracting agent might be an organic compound. To elucidate this point the following procedures and tests were carried out. Muscle extracts dialyzed through cellophane bags were found to have lost their activity for melanophores. Although this would eliminate organic compounds of large molecular weight, such substances as adrenalin are readily dialyzable (*3*). On the other hand, boiling extracts and keeping them in an unsterile state for 2 weeks did not reduce their potency. As a further, and crucial, procedure a muscle extract was incinerated, water was added, and the H-ion concentration adjusted to bring it back to its original volume and reaction (pH 8.0). This reconstituted extract exhibited fully as great melanophore-contracting activity as did that part of the same extract kept untreated as a control; hence there seems to be no doubt that the active principle is an inorganic compound.

Further studies showed that slices of trout brain, liver, and spleen, tissues known to be relatively rich in potassium, all possessed the property of producing contraction of the melanophores of skin strips. Fresh beef muscle also exhibited the same effect, whereas blood serum, which contains approximately 5 mEq potassium/l, and a ratio of Na to K of about 30 : 1, caused no change in the state of the pigment cells. These findings add support to the probability that the melanophore concentration and attendant pallor of the trout's skin resulting from pressure are due to liberation of potassium from the underlying muscle.

[2] The author wishes to express his appreciation to Lillian Eichelberger, of the Department of Surgery, University of Chicago, for her kindness in carrying out these chemical determinations, and to E. L. Tatum, Department of Biological Sciences, Stanford University, for suggestions concerning the other analytical procedures.

References

1. ROBERTSON, O. H. *Physiol. Zoöl.* (in press).
2. FRISCH, K. VON. *Arch. ges. Physiol.*, **138**, 319 (1911).
3. PARKER, J. H. *Proc. Am. Phil. Soc.*, **83**, 379 (1940).

Fluorometric Determination of Nicotinamide by Use of Synthetic Ion Exchange Resins

Masaru Kato and Hiroshi Shimizu[1]

School of Textile Fibers, Kyoto Technical University, and Department of Industrial Chemistry, Kyoto University, Kyoto, Japan

Chaudhuri and Kodicek (*1*) have reported a fluorometric determination of nicotinamide by treatment with cyanogen bromide solution under specified conditions. In that case, interferences of the other fluorescent substances were practically eliminated by the use

[1] The authors are indebted to K. Nakamura, M. Fujiwara, H. Kikkawa, R. Oda, Y. Hamamura, and S. Kobayashi.

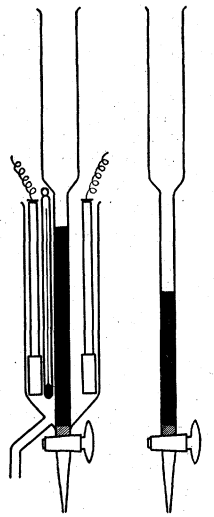

FIG. 1. Exchange tubes (col diam, 7 mm). Tube A, left, KH-4B-Na; tube B, right, Amberlite IRA-400-OH.

of acid and alkaline pretreatment and a special blank, but complete elimination of the interferences was not performed by the method.

We have found that fluorescences caused by kynurenine, 3-hydroxykynurenine, and the other unknown substances in the silkworm *Bombyx mori* cannot be eliminated by the usual methods, but they can be eliminated completely by successive purification with carboxylic-type cation exchange resin KH-4B (*2*) and strong-base–type anion exchange resin Amberlite IRA-400 (*3*).

Reagents used:

Synthetic cation exchange resin KH-4B (Oda Laboratory of Kyoto University).

Synthetic anion exchange resin Amberlite IRA-400 (Rohm & Haas Co.); 25% metaphosphoric acid solution, freshly prepared at the time of using.

CNBr solution, freshly prepared just before the experiment by adding ice-cold 10% aqueous NaCN solution by drop to ice-cold saturated bromine water until it is just decolorized.

KH_2PO_4-NaOH buffer solution (pH 7).

5% NaCl solution (pH 5).

0.2*N* NaOH-N KCl.

1*N* NaOH and 15% NaOH solution.

0.1*N*, 1*N*, 5*N* HCl.

Standard nicotinamide solution (5γ/ml), prepared weekly from a stronger nicotinamide solution.

Apparatus. Two exchange tubes (Fig. 1) are used for purification of the extract solution. Tube A, filled with 1 g KH-4B, is used for removal of interfering cations, and Tube B, filled with 3 ml Amberlite IRA-400, is used for removal of interfering anions.

Tube A is treated with 100 ml hot 0.2*N* NaOH-N KCl at the rate of 1 ml/min, 100 ml hot distilled water at the rate of about 20 ml/min, 200 ml hot 5% NaCl solution (pH 5) at the rate of 3 ml/min, and then 100 ml hot distilled water at the same flow rate. The jacket is used to maintain temperature of the resin bed at 80°–85° C throughout these pretreatments. Tube A is then treated with 70 ml cold distilled water without the use of the jacket, at the rate of about 20 ml/min. Tube A is then ready for use. Tube B is treated with *N* HCl at the rate of 1 ml/min, 100 ml distilled water at the rate of 20 ml/min, 100 ml *N* NaOH at the rate of 1 ml/min, and is then rinsed with about 100 ml distilled water at the rate of 3 ml/min until pH of the filtrate has become neutral. Tube B is then ready for use.

Procedure. The material to be tested (5 g) is weighed out, cut up fine (with scissors if necessary), and ground with a small amount of 0.1*N* HCl (1–2 ml). The ground tissue is transferred with 30 ml water into a 50-ml centrifugal tube and heated in a boiling water bath for 30 min. The solution is cooled, brought to pH 2 with concentrated hydrochloric acid, centrifuged for 10 min, and the residue washed with 10 ml 0.1*N* HCl and centrifuged. The combined centrifugates are brought to pH 5 and centrifuged for 10 min without agitation after standing for 5–10 minutes. Volume of the supernatant solution is measured and is used as an extract solution.

Six ml of the extract solution is placed in a 20-ml beaker, and 4 ml distilled water is added (sample for determination). Six ml of the same extract solution is placed in another 20-ml beaker, and 4 ml nicotinamide solution (5γ/ml) is added (sample of intermediate standard). These two sample solutions are each allowed to pass through tube A at the rate of 1 ml/min and washed with 20 ml hot distilled water at the same flow rate at 80°–85° C, using the jacket. Each combined filtrate (30 ml) is cooled to room temperature, adjusted to pH 5, then allowed to pass through tube B at the rate of 1 ml/min, and rinsed with 40 ml distilled water at the same flow rate. Each combined filtrate (70 ml) is used for the next procedure.

Forty ml of each filtrate is placed in two centrifugal tubes, to which is added 6 ml freshly prepared 25% metaphosphoric acid, and centrifuged after standing for 5–10 min at room temperature. The clear solutions are brought to pH 9.4–9.6 (thymol blue as external indicator) and heated in beakers in a boiling water bath for 30 min. The solutions are cooled, adjusted to pH 7, and the volume made up to 100 ml with KH_2PO_4-NaOH buffer solution (pH 7). They are then ready for the next stage.

One ml of each solution is placed in nonfluorescent test tubes, and 2 ml of KH_2PO_4-NaOH buffer solution (pH 7) and 1 ml of freshly prepared CNBr solutions are added to each tube. Blank solution is prepared in the same manner except that 1 ml distilled water is used in place of 1 ml CNBr solution. These three samples are heated in a water bath at 70°–80° C for 8 min, cooled in ice-cold water, and 1 ml 15% NaOH solution is added to each. After standing for 30 min at room temperature in the dark, fluorescences are measured, comparing with the blank by the use of a fluorometer or by titration, using standard fluorescent solution prepared from standard nicotinamide solutions (5γ/ml). Standard fluorescent solution is prepared as follows: 5 ml standard nicotinamide solution

(5γ/ml) is added with 10 ml KH_2PO_4-NaOH buffer solution (pH 7) and 5 ml CNBr solution, and heated in a water bath at 70°–80° C for 8 min, cooled in ice-cold water, and 5 ml 15% NaOH solution added.

Calculation. Let a = content of nicotinamide in the sample for determination; b = content of nicotinamide in the sample of intermediate standard; c = amount of nicotinamide added to the sample for determination; f = dilution factor; and r = recovery (%) of the added nicotinamide throughout the operation. Then

$$\text{Nicotinamide } (\gamma/\text{g}) = \frac{acf}{b-a}$$

$$\text{Recovery } (\%) = \frac{b-a}{c} \times 100.$$

KH-4B is a carboxylic-type cation exchange resin prepared from phenoxyacetic acid and formaldehyde, and has a total capacity of 5.84 mEq/g dry resin. The same types of cation exchange resins, Amberlite IRC-50 (Rohm & Haas Co.) (*4*) and Wofatit C (I. G. Farbenindustrie Akt.-Ges.) (*5*) may be suitable for use in place of KH-4B. Amberlite IRA-400 is a strong-base–type anion exchange resin, and has a total capacity of 2.5 mEq/g dry resin. Since the chemical characteristics of Amberlite IRA-400 are analogous to those of sodium hydroxide, carbonate-free reagents and distilled water must be used for the treatment.

Cationic impurities in an extract solution are adsorbed by the filtration through KH-4B-Na (sodium salt–type of KH-4B) at pH 5, but nicotinamide is not adsorbed by such an operation followed by the rinse with hot distilled water. Anionic impurities in an extract solution are adsorbed by filtration through Amberlite IRA-400-OH (hydroxide-type of Amberlite IRA-400) at pH 5, but nicotinamide is not adsorbed by such an operation followed by the rinse with distilled water.

Nicotinamide contents of the pupae and eggs of various types of silkworm *B. mori* were determined by the method described above, and the analytical results shown in Table 1 were obtained.

TABLE 1

NICOTINAMIDE CONTENT OF SILKWORM

Material	Nicotinamide content (γ/g)	Material	Nicotinamide content (γ/g)
Pupa of White-1 type	59	Egg of White-1 type	111
" " White-2 "	13	" " White-2 "	30
" " normal "	60	" " normal "	113

By Chaudhuri-Kedicek's method, kynurenine in the White-1 type mutant (*6*) and 3-hydroxy kynurenine in the White-2 type mutant (*6*) gave green and yellowish-green fluorescence, respectively, and unknown substances other than nicotinamide in each type of *B. mori* gave yellowish fluorescence after treatment with cyanogen bromide. Therefore the fluorescence of nicotinamide was greatly contaminated and the estimation was almost impossible. But in our method these contaminating fluorescences were eliminated completely by the use of KH-4B-Na and Amberlite IRA-400-OH, and the estimation of nicotinamide was performed without difficulty.

The new determination method of nicotinamide by use of synthetic ion exchange resins as described should be especially useful in the investigation of tryptophan metabolism in future.

References

1. CHAUDHURI, D. K., and KODICEK, E. *Biochem. J.*, **44**, 343 (1949).
2. SHIMIZU, H. *The Chemistry of High Polymers* (in press).
3. KUNIN, R., and MCGARVEY, F. X., *Ind. Eng. Chem.*, **41**, 1265 (1949).
4. KUNIN, R., and BARRY, R. H. *Ibid.*, 1269.
5. GRIESSBACH, R. *Angew. Chem.* **52**, 215 (1939).
6. KIKKAWA, H. *Sanshi Shikenjô Hôkoku* (in Japanese), **11**, 311 (1943).

The Use of K^{42}-tagged Erythrocytes in Blood Volume Determinations[1]

Rosalyn S. Yalow and Solomon A. Berson[2]

Radioisotope Unit,
Veterans Administration Hospital, Bronx, New York

Erythrocytes have been tagged with $Fe^{55, 59}$ (*1*), P^{32} (*2, 3*), and Cr^{51} (*4*) and used for blood volume determinations by the *in vivo* dilution technique. Radiopotassium (K^{42}) has two properties that make it useful for tagging erythrocytes for special types of experiments. First, it decays by emission of energetic β-particles (3.6 and 2.0 mev) and γ-rays, which are easily detectable in liquid samples. Therefore extremely small amounts (less than 2 μc) may be used effectively. A second advantage is that it has a short physical half-life of 12.44 hr. Consequently, blood volume determinations or other studies may be repeated at relatively short intervals without concern for residual activity or hazards created by the concentration of radioisotopes in any part of the body. Furthermore, isotopes such as P^{32} and I^{131}, with appreciably longer half-lives, may be administered shortly thereafter. Because of the great difference in half-lives, the activity of the longer-lived isotope in body fluids can be determined after the K^{42} has decayed, without the need for complicated corrections.

This paper describes experiments in which comparison studies were made between almost simultaneous blood volume determinations with erythrocytes labeled with K^{42} and P^{32}. The *in vivo* half-life of the circu-

[1] Reviewed in the Veterans Administration and published with the approval of the chief medical director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or the policy of the Veterans Administration.

[2] The authors are greatly indebted to K. Newerly, biochemist of the radioisotope laboratory, for her cooperation and technical assistance in this experiment. Grateful acknowledgment is also extended to Bernard Roswit, director of the Radioisotope Unit, for making this work possible.