THE BLOOD V-FACTOR (COENZYME) LEVEL IN NORMAL AND PATHOLOGICAL SUBJECTS [1]

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(Received for publication May 10, 1939)

The fundamental importance of the nicotinic acid-containing coenzymes [2] in cellular oxidations and reductions is now generally accepted, and much detailed information is available concerning the reactions in which they are involved (1, 2). As yet, however, only a beginning has been made in analyzing the factors which control the coenzyme level in the tissues. The most important is nicotinic acid itself, which is a vitamin for the dog, man, ape, and pig (3, 4, 5, 6, 7).

The relation between nicotinic acid and coenzyme has been examined in man and the dog. In the human, whether normal or pellagrin, a dose of the acid (*e.g.* 20 mgm. per kilo) leads to a large increase in blood coenzyme; the increase, however, is transient, suggesting the existence of a homeostatic mechanism (8). The phenomenon is a corpuscular one and can be obtained *in vitro* where normally it is limited only by the presence or absence of the acid (9). Other factors are made to limit the *in vitro* synthesis, however, if the subject furnishing the blood is treated previously with nicotinic acid.

In pellagra, the coenzyme level may be somewhat low. The discrepancy between the results of Kohn (8) who found relatively small differences and of Vilter, Vilter, and Spies (10) who claim the difference is striking, is at least partly explained by errors in the method used by the latter authors. This question will be discussed later. In their more recent publication (11), they have presented evidence indicating that the diabetic level is low.

In the dog, no information can be obtained from a study of the blood coenzyme level, since it is not affected in black tongue or by nicotinic acid (12, 13). In their preliminary report, however, Axelrod and Elvehjem (12) state that muscle and liver were deficient in *DPN* [2] in a dog with black tongue.

The present work is a further attempt to determine the factors which influence the blood coenzyme level in the human by examining a variety of normal and pathological subjects. From a study of some 244 cases it appears that high levels are correlated with pneumonia, low levels with diabetes, to some extent with pellagra, and with some conditions which as yet cannot be accurately defined. The general distribution in the population is such, however, that little practical importance can be attached to the blood coenzyme level.

METHODS

Lwoff and Lwoff (14) found that the *V*-factor required for the growth of *Haemophilus para-influenzae* can be replaced by either *DPN* [2] or *TPN*,[2] and Kohn (8) has developed a method for the bio-assay of the coenzymes based upon this fact. As far as is known, the method is specific for the coenzymes,[3] although it does not distinguish between them. The principle is to determine the volume of corpuscles (to which the *V*-factor is confined) necessary to produce a bacterial turbidity equal to that produced by a standard growth factor solution.

Vilter, Vilter, and Spies (10) described a procedure [4] for the bio-assay of blood *V*-factor, stating (11) that Kohn (8) "has published recently a method similar to ours." We would like to emphasize four major differences. (*1*) They employ *H. influenzae*, whereas we use the same strain of *H. para-influenzae* employed by the Lwoffs. (*2*) Their extract is prepared by heating diluted blood under conditions which may lead to a variable loss of activity. (*3*) They determine the least amount of blood extract producing a visible growth, using serial dilutions differing by 50 per cent or more (1/12,000, 1/8,000, 1/4,000, etc.). Our method involves a quantita-

[1] Part of the cost of this investigation was defrayed by a grant from the Duke Research Council.

[2] Diphosphopyridine nucleotide (coenzyme one, cozymase, designated *DPN*) is active in the oxidation of lactic acid, the reduction of acetaldehyde to alcohol, etc. Triphosphopyridine nucleotide (coenzyme two, designated *TPN*) participates in the oxidation of glucose after the latter has been phosphorylated.

[3] We have found recently that 1 mgm. of pure riboflavin, of thiamin hydrochloride, and of cevitamic acid are without effect when added to the acid extract of blood.

[4] This procedure is essentially that of Lwoff and Lwoff (14) who determined the approximate *V*-factor content of rabbit blood while proving that either *DPN* or *TPN* could serve as *V*-factor.

tive determination in the photoelectric densitometer (Evelyn) of the amount of unknown necessary to produce a turbidity equal to that produced by a standard extract. The standard deviation in any series is 7.2 per cent. (*4*) They calculate their results *per volume of blood,* whereas we calculate ours *per volume of corpuscles.* Since blood *V*-factor is confined to the corpuscles, as shown for the *DPN* moiety by Euler and Nilsson (15) and for the entire *V*-factor by Kohn (8), comparison of the various assays of Vilter, Vilter, and Spies (10) must be inaccurate to at least the extent by which the hematocrits differ—which in ordinary hospital work may easily be 100 per cent.

The results obtained are expressed in *d.e* or *DPN equivalents.* An assay of 10 *d.e.* signifies that 1 ml. of corpuscles has a growth promoting activity equal to that of 10 gamma of *DPN* (cozymase). It is to be emphasized, however, that this is an arbitrary measure, since the relative contribution made by each coenzyme (and possibly by unknown related substances) has not been determined. The sensitivity of the method is such that under the usual conditions visible growth is obtained at a dilution of cozymase about 1 part in 500 million, or more. The quantitative estimations can be carried out at about half this dilution with a standard deviation of about 7 per cent in any series. We are greatly indebted to Prof. J. Rünnström and Drs. A. Lennerstrand and E. Sperber of Stockholm for a generous gift of *DPN* (63 per cent purity) with which we have calibrated our standards.

Since the specificity, theory, and procedure of the test have already been discussed in detail (8), it is only necessary to give here a résumé of our technic, indicating the improvements which have been introduced. It should also be noted that the pH of the blood extract is such that the reduced form of the coenzymes is inactivated. The amount of the reduced form present, however, is usually less than 10 per cent, when estimated as the increase in assay following the addition of $K_3Fe(CN)_6$ to the laked cells previous to trichloracetic acid precipitation.

Blood is obtained by puncturing the finger tip with a surgical blade (Crescent 111) and gently pressing out 4 or 5 drops into the depression of a small paraffin block; 0.2 ml. are then added to 0.8 ml. of isotonic saline (17 volumes of 0.9 per cent NaCl + 1 volume of 2.2 per cent $K_2C_2O_4.H_2O$). Such diluted blood can be kept at room temperature for 2 hours or at 5° for 5 hours without loss of coenzyme.

For the hematocrit, 0.4 ml. of diluted blood are centrifuged in a special pipette, 13.5 cm. long, illustrated in Figure 1a. The walls are of pyrex, about 3 mm. thick, and the bore of the graduated tube is of the order of 1 mm. After filling, the ends are wiped dry, each is sealed with a small piece of adhesive tape, and the whole pipette is enclosed within a rubber band (Goodrich Number 84), the ends being taped as in Figure 1b. The rubber band acts as a shock absorber and permits a half dozen pipettes

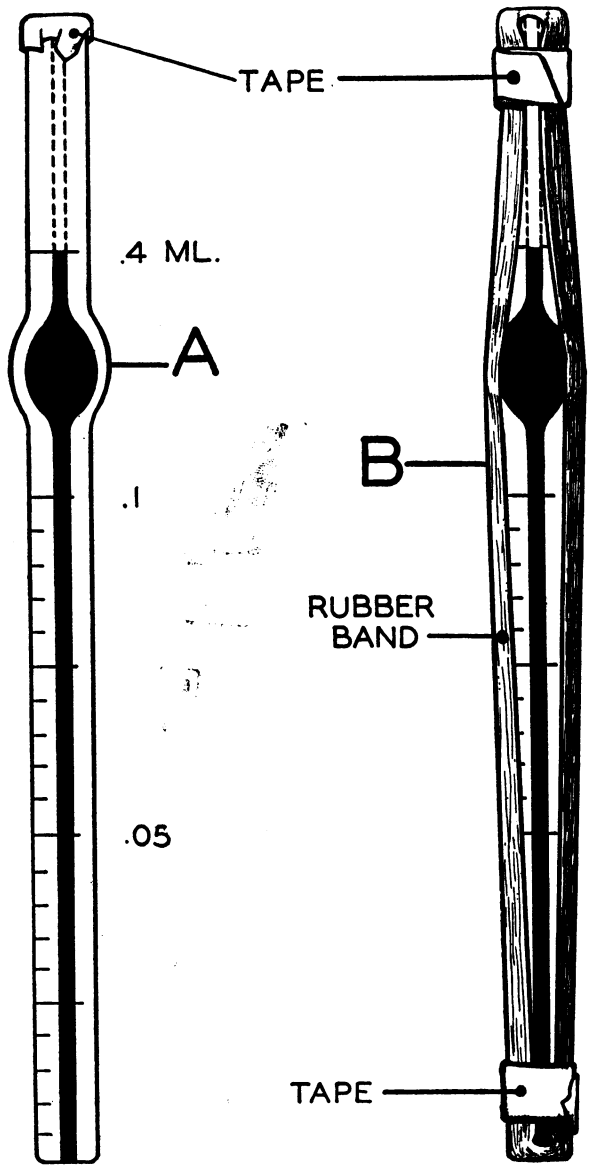

FIG. 1. THE HEMATOCRIT PIPETTE

After filling, the ends are wiped dry and each is sealed with a small piece of adhesive tape as shown at the top of *A*. The pipette is then encased in a rubber band which is held in place by two rings of tape, as in *B*.

to be spun in one large centrifuge cup. After centrifuging at 2000 r.p.m. for 15 minutes, the volume of the packed cells is read directly from the calibrated stem. These cells can be used for analysis, if desired, by removing the tape and blowing out the contents of the pipette. Ordinarily, however, an aliquot of diluted blood is used for the blood extract.

Blood extract is prepared by laking 0.2 ml. (or more, if necessary) of diluted blood in 7.8 ml. of distilled water and adding within several minutes 2 ml. of partially neutralized trichloracetic acid. The test tube is then corked, inverted (to sterilize), centrifuged, and stored in the cold. The clear (or almost clear) supernatent liquid is tested. The strength of the trichloracetic acid used is about 13.5 per cent, and its pH is 2.1 to 2.2 due to the addition of NaOH. After its addition to the laked blood, the pH of the extract is about 3, which gives optimal protein precipitation.

The broth employed for both stock cultures and tests contains 2 per cent proteose peptone, 0.6 per cent NaCl, 0.1 per cent sucrose, and 0.04 per cent fumaric acid, titrated with NaOH to pH 7.8 (glass electrode). To prevent unnecessary darkening, it is autoclaved for not more than 15 minutes at 15 pounds pressure. It is superior to the broth used formerly in that it permits a comparison of the unknown against the standard at an a_s (turbidity) of 15, or less, with a single reading 20 to 24 hours after inoculation. For clinical determinations, only the standards need be made up in duplicate. The stock cultures are transferred daily. For every assay, to a series of tubes each containing 7 ml. of broth, there are added 0.0, 0.1, 0.2, and 0.3 ml. of blood extract. The tubes are then inoculated, and the turbidity is finally determined and compared with that produced by a standard *V*-factor solution as previously described in detail (8).

RESULTS

The blood coenzyme (*d.e.*) values for a group of 126 hospital patients, selected for the most part at random, were determined and compared with those for a group of 53 normals. The results are summarized in Figure 2 in the form of frequency distribution polygons which show the percentage of cases having a *d.e.* of 50 to 60, of 60 to 70, of 70 to 80, etc. It is apparent that the *d.e.* values of the clinical subjects have a greater spread than the normal, although the mode of each group falls in about the same range. Of the controls, 64 per cent fall between 50 and 70 *d.e.* and 49 per cent between 60 and 80 *d.e.;* of the clinical subjects, 47 per cent fall into each of these classes. The peak between 60 and 70 *d.e.* in the clinical distribution, which is not seen in the controls, may be due to the greater number of females in the clinical series (46 per cent compared to 15 per cent). The female mode falls between 60 and 80 *d.e.;* whereas that for the males lies between 50 and 70 *d.e.*

The greater spread of the *d.e.* values in the hospital population indicates that the normal distribution has been distorted by an increase in the relative number of high and low values. We have found that diabetics tend to be low and patients suffering from pulmonary disease (pneumonia) tend to be high, whereas cases of cardiovascular disease tend to have a normal distribution. Probably, most other conditions have essentially normal distributions since, on the whole, the polygon for the clinical subjects is similar to that for the controls. Similar results were obtained in an earlier series of 65 cases, which are not reported, however, since the technic employed was somewhat different.

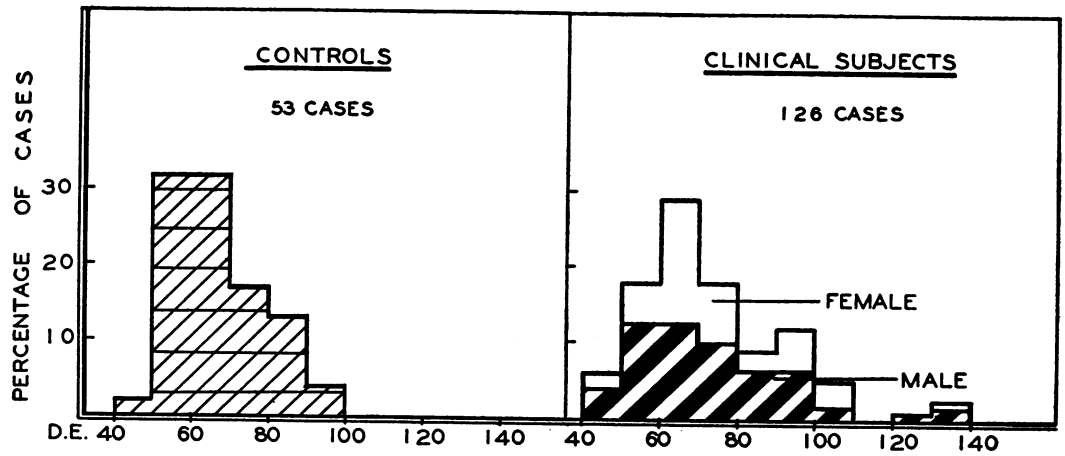

FIG. 2. THE DISTRIBUTION OF *V*-FACTOR (*d.e.*) LEVELS IN NORMAL AND PATHOLOGICAL SUBJECTS

Diabetes mellitus

The distribution of 23 cases is shown in Figure 3, where 2 peaks are seen. Thirty per cent of the cases have significantly low values of 40 to 50 *d.e.* About 50 per cent fall in the definitely normal range of 50 to 80, while the remainder tend to be high. Vilter, Vilter, and Spies (11) examined 3 diabetics in acidosis and found their initial values to be low; when 2 of them were treated with large quantities of insulin and were given other routine therapy, an increase in assay of well over 100 per cent was noted within 10 days. Red cell counts were around 5,000,000. It was therefore possible that the peaks in our series could be correlated with the amount of treatment received. This, however, was not the case. The average *d.e.* for 11 patients who had received no insulin was 55, which was within a few per cent of the average of the insulin-treated cases. Further examination of the individual data revealed no connection between insulin therapy or blood sugar concentration and *d.e.* level. In 2 cases there was actually a drop of 20 to 25 per cent in blood *d.e* after 10 days of insulin administration. Our results show, therefore, that the diabetic population has a larger percentage of low *d.e.* values than the normal, which is consistent with the findings of Vilter, Vilter, and Spies. The differences noted by us, however, are smaller, and the administration of insulin seems to be unaccompanied by any dramatic effect. This may be accounted for by the fact that we have not examined diabetics during and after recovery from extreme acidosis. Also, the growth factor specificity of the *para-influenza* bacillus may differ from that of the *influenza bacillus* used by Vilter *et al.*

Pulmonary disease

The distribution of 15 cases is shown in Figure 3. The tendency to high *d.e.* values in this group is due to the cases of pneumonitis (cross hatched) rather than to those of tuberculosis (solid). All of the pneumonitis cases except one had been treated with sulfapyridine for at least several days before the blood tests were made. The temperatures had dropped to around 38° and the patients were on the way to recovery. The untreated case was one of bronchopneumonia, had a temperature of 40°, and a *d.e.* of 79. There are also included with this group 1 case of pleural effusion and 1 of lung abscess. All of the tuberculosis cases were active.

Pellagra

Four pellagrins tested before treatment were reported (8) to be within 20 per cent of 6 normals, the assays being expressed in arbitrary units. The assays converted into *d.e.,* on the basis of re-testing three of the normals, are 50, 50, 54, and 68. Recalculated *d.e.* values for 3 other pellagrins, not previously reported, are: adult female, moderately ill, 61; adult male, acutely ill, 52; 10-year-old boy, acutely ill, 70. The tests were made before nicotinic acid was given and while the patients were still sensitive to sunlight. Two more cases are included in the present series, both adult males, a very mild one, *d.e.* 64, and a severe one, *d.e.* 55. All of these results are consistent with the conclusion that pellagrins are only moderately low, falling in the lower range of the normal distribution. The very low values found by Vilter *et al.* (10) may be explained, at least in part, by the error in their method of comparison (*cf.* Methods). It is also possible that other differences in technic, particularly the use of another species of bacterium, may lead to divergent results.

Cardiovascular disease and others of normal distribution

The distribution of 15 cases of cardiovascular disease is shown in Figure 3. Other groups which appeared normal in distribution were 8 cases of gastro-intestinal disorders (including ulcers and cholecystitis), 8 of psychoses of various types, 5 of syphilis, and 4 of thyroid dysfunction (3 hyper, 1 hypo). Vilter *et al.* (11) reported an extremely low value for a case of chronic lymphatic leukemia. The 3 cases examined by us were not definitely abnormal.

Miscellaneous cases

It is impossible to draw conclusions concerning the other diseases where only 1 or 2 cases were examined. However, 2 cases were found with definitely abnormal *d.e.* values in the vicinity of 30. They have not been included in the above sta-

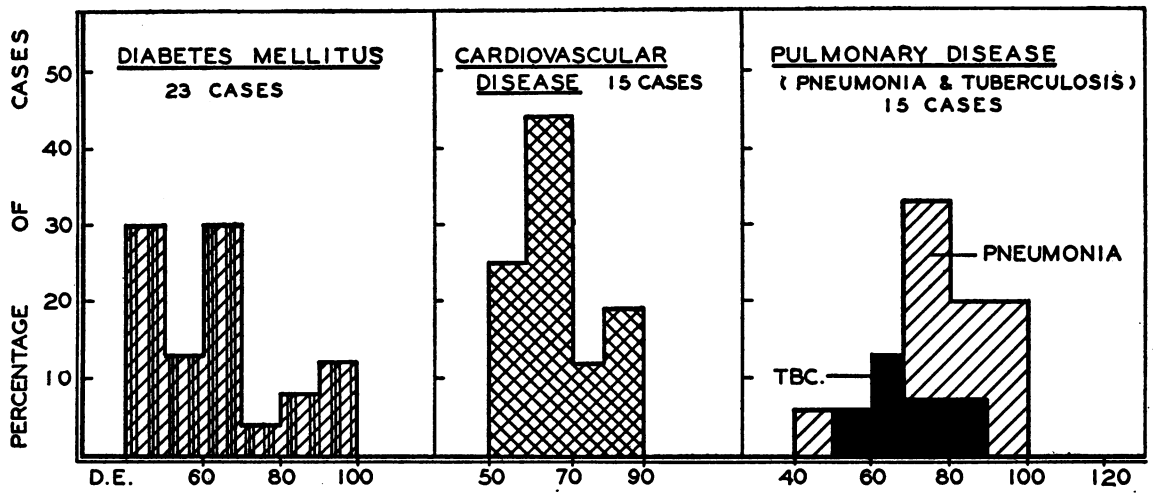

FIG. 3. THE DISTRIBUTION OF *V*-FACTOR (*d.e.*) LEVELS IN DIABETES MELLITUS, CARDIOVASCULAR DISEASE, AND PULMONARY DISEASE

The cardiovascular disease shows a normal distribution, the diabetes shows a significant percentage of low values, and the pulmonary disease a significant percentage of high values.

tistics. Both were women, the first having a generalized type of osteomalacia which was refractory to all treatment, and the second having sprue. The low value in the sprue case is not characteristic, since 2 others showed *d.e.* values of 84 and 57 respectively. Both responded as readily and in the same way as normals and pellagrins to the administration of nicotinic acid, the *d.e.* value increasing during administration, but falling back when dosage was discontinued. Although the value could be raised far beyond the normal, no clinical improvement resulted. Like the diabetics, these cases show that low *d.e.* values are possible in conditions which are not specifically improved by nicotinic acid, and emphasize the discreteness and independence of the factors which control the *d.e.* level. In this connection, the case of a normal individual is worth mentioning. We have found the *d.e.* level to be quite constant when followed for fairly long periods of time (several months). A striking exception to this is shown in Figure 4, where 2 healthy, adult males are compared over a period of 65 days. The variation of Subject *A* who served as a control was about $\pm$ 3 per cent, whereas Subject *B* varied between 84 and 63 *d.e.* Nevertheless Subject *B* enjoyed excellent health and his diet and activities experienced no unusual variation.

Among the hospital patients in Figure 2 there is a small group which falls between 120 to 140 *d.e.* This includes 1 case of pernicious anemia (131 *d.e.*) which had received 30 U.S.P. units of liver (Lederle) intramuscularly daily for 7 days. During this period the erythrocyte count rose from 1.4 to 1.85 million, and the reticulocyte count rose from 0.2 to 29 per cent. On the basis of other studies (9), it is most probable that the high reticulocyte count is responsible for the high assay in this case. The other 2 cases cannot be

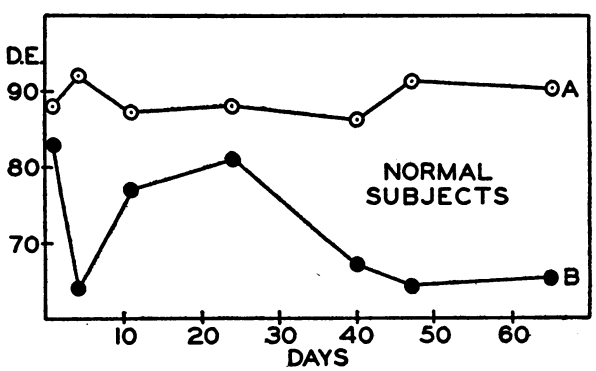

FIG. 4. THE *V*-FACTOR (*d.e.*) LEVELS IN TWO NORMAL INDIVIDUALS

A is typical in showing a relatively constant level. *B* is atypical, showing a marked variation which is not correlated with any change in condition.

explained. One was diagnosed as epilepsy probably of organic origin (125 *d.e*), the other was one in which the final clinical impression was menopausal syndrome and malnutrition. (134 *d.e.*) For comparison, it may be noted that a case of idiopathic epilepsy had an assay of 62 *d.e.*

DISCUSSION

The present work emphasizes the great variability in *d.e.* existing in a population of normal individuals, differences of the order of 50 per cent being found frequently. The small variation previously reported (8) in a group of 6 individuals was probably due to homogeneity with respect to age, sex, and occupation. In spite of this variation, however, we now know of one factor which directly affects the *V*-factor level, namely, nicotinic acid; of two associated with depression, diabetes mellitus, and pellagra; and of one associated with elevation, pulmonary infection.

In addition, however, it seems necessary to postulate at least one other factor which operates independently of the above in order to account for (*a*) the extremely high and low assays, (*b*) the normal variation, and (*c*) the type of instability shown by Subject *B*, Figure 4. The relative stability of the blood level (which is extreme in Subject *A*, Figure 4) was noted previously (8) and formed the basis for the suggestion that it is determined by the general condition and relationships of the hematopoietic mechanism. On the other hand, the increment following nicotinic acid dosage (and which disappears when dosage is discontinued) can be accounted for as a corpuscular phenomenon and can be duplicated *in vitro* (9). Such an hypothesis implies that the blood level depends not only upon the current state of nutrition, but also upon the condition of the myeloid apparatus, and upon its power of abstracting needed substances from other loci. To judge by our results, the nutritional factor is of some but not of great importance in man; and the same is true of the dog. It also seems that considerable latitude is possible in the relations involved without danger to the organism. These considerations lead to the obvious conclusion that the *d.e.* level, as determined by our technic, can be of little importance for diagnosis or prognosis.

SUMMARY

The *V*-factor (coenzymes one and two, possibly plus unknown related substances) level was determined for the blood of 53 normal individuals and 126 hospital patients. The assays are expressed in *d.e.* or activity *equivalent* to: gamma of coenzyme one per ml. of corpuscles. Eighty-one per cent of the normal cases fell between 50 and 80 *d.e.* whereas only 65 per cent of the pathological cases fell in this range. The greater variation in the pathological series was caused, to a large extent, by the diabetics who showed low values and the patients with pulmonary disease who showed high values. Pellagrins showed values that were in the lower part of the normal range. To account for all of the variation observed, it is necessary to postulate factors other than diet or disease which regulate the blood *d.e.* An earlier series of 65 cases, not reported in detail, is consistent with these conclusions.

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