

High-Purity 4-Nitrophenol: Purification, Characterization, and Specifications for Use as a Spectrophotometric Reference Material

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We describe specifications for high-purity 4-nitrophenol, which is suitable for spectrophotometric standardization. Such a reference material is needed in clinical enzymology to establish the proper molar absorptivity of 4-nitrophenol under final reaction conditions, particularly for measuring alkaline phosphatase activity in human serum. Some lots of 4-nitrophenol available commercially met these specifications, but several did not. The latter can be purified to meet our specifications by recrystallization or sublimation. The molar absorptivity of 4-nitrophenol (35 $\mu\text{mol/L}$) in 10 mmol/L NaOH at 25 °C at 401 nm is $18\,380 \pm 90 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$.

Additional Keyphrases: molar absorptivity (molar absorption coefficient) • enzyme activity

4-Nitrophenol (4NP, also "*p*-nitrophenol")³ is an important chemical material in biology and medicine. This highly chromogenic, stable compound is the product of enzyme catalysis of any of several synthetic substrates (Table 1) (1). One of these synthetic substrates, 4-nitrophenyl phosphate (4NPP), is of particular interest in clinical chemistry for the measurement of alkaline phosphatase (EC 3.1.3.1; ortho-phosphoric-monoester phosphohydrolase, alkaline optimum) activity in human serum or other body fluids or tissues (2).

The spectral absorbance curves of 4NP and 4NPP in an alkaline solution are given in Figure 1. The molar absorptivity of 4NP near 400 nm has been reported to be 18 000 to 20 000 $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$, depending upon the composition of the solution and the physical conditions (Table 2) (3-11). This high but variable value for 4NP stands in marked contrast to the extremely low molar absorptivity of its phosphate ester 4NPP, which is about 50 $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at this same wavelength (12). These desirable spectral characteristics of 4NP and 4NPP, coupled with the high activity observed when alkaline phosphatases of human serum hydrolyze 4NPP, have made it one of the most widely used phosphatase substrates (2). As the number of different methods involving 4NPP proliferates (2, 13-21), the need for highly purified 4NPP and 4NP as certified reference materials has become ever more apparent. These key reference materials, particularly 4NP, are necessary if we are rationally to interrelate commonly used and newly appearing methods with the different reference methods for alkaline phosphatase promulgated in Germany (13), Scandinavia (14), France (19), and the United States (20).

As shown in Table 3, the final reaction conditions of the many alkaline phosphatase methods involving 4NPP as a substrate differ markedly with respect to (a) buffer type and concentration, (b) final reaction pH, (c) reaction temperature,

(d) sample size and total reaction volume, (e) magnesium concentration, and (f) order of reagent and sample addition. As a result of these differences, the upper reference limit of activity for this enzyme in serum of adults can range from 80 to 375 U/L.

Note also that the molar absorptivity of 4NP changes, depending upon the solution environment into which it is released. Figure 2 illustrates how temperature (from 25 to 37 °C) and three different alkaline conditions affect the molar absorptivity of 4NP (10). Lehmann et al. (11) called attention to the fact that components of the final reaction mixture other than the buffer (i.e., albumin, 4NPP, and magnesium) can also affect the absorbance of 4NP in the final reaction mixture. Other workers have described the difficulty of making accurate spectrophotometric measurement of 4NP in the presence of large and changing concentrations of 4NPP (22-24). Resolution of these various problems requires the availability of well-characterized high-purity 4NP.

Reported Values for 4NP and Tests of Commercial Materials

Table 2 lists reported molar absorptivity values for alkaline solutions of 4NP near 400 nm (3-11). Although these measurements have been reported over a span of three decades and were made by many workers who used many different instruments and different solvents, the spread between the high and low values of 18 300 to 20 000 is surprisingly small considering the number of variables involved. Even so, when conditions are limited to a NaOH matrix at 25 °C, the range narrows to 18 330 to 18 400 $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$, which suggests that it is easy to obtain relatively pure 4NP. Although our long experience with the analysis of 4NP confirms that materials sold specifically as "colorimetric standard," "spectrophotometer standard," or "99%" are usually satisfactory,⁴ we must still caution that poor materials are also available commercially. The absorbance of only four of the materials studied at Hartford were equal to that of high-purity 4NP; the absorbance of seven other materials fell between 97 to 99%. However, the absorbance of six other materials fell between 70 to 94%! Hence, we recommend that before 4NP from any source is used as a primary reference material for purposes of standardization or calibration, the specifications given below be either certified by the supplier or met by actual test in the user's laboratory.

Purification Procedures

Recrystallization. One recrystallization from hot water according to simple instructions given in a laboratory text book on organic chemistry (e.g., ref. 25) markedly improves impure material, such as sample 7 of Table 4. Multiple recrystallizations from water are better. The reference material described by Bowers et al. in 1978 (10) was purified from practical grade 4NP (No. P192; Eastman Kodak Co., Roch-

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³Nonstandard abbreviations used: 4NP, 4-nitrophenol; 4NPP, 4-nitrophenyl phosphate; NBS, National Bureau of Standards; and SRM, Standard Reference Materials.

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⁴Identification of any commercial product does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the material or equipment identified is necessarily the best available for the purpose.

Table 1. 4-Nitrophenol-labeled Substituents Yielding 4-Nitrophenol after Hydrolysis with Indicated Enzyme

Substrate (4-nitrophenol substituents)	Enzyme and EC no.
Acetate	Arylesterase, 3.1.1.2
<i>N</i> -Acetyl- β -galactos- amide	β - <i>N</i> -Acetylglucosaminidase, 3.2.1.53
α -L-Arabinofuranoside	α -L-Arabinofuranosidase, 3.2.1.55
Butyrate	Cholinesterase, 3.1.1.8
<i>N</i> -Carbobenzoxy-L-tyro- sine	Chymotrypsin, 3.4.21.1
α -L-Fucoside	α -L-Fucosidase, 3.2.1.51
α -D-Galactoside	α -Galactosidase, 3.2.1.22
β -D-Galactoside	β -Galactosidase, 3.2.1.23
α -D-Glucoside	α -Glucosidase, 3.2.1.20
β -D-Glucoside	β -Glucosidase, 3.2.1.21
β -D-Glucuronide	β -Glucuronidase, 3.2.1.31
α -D-Mannoside	α -Mannosidase, 3.2.1.24
β -D-Mannoside	β -Mannosidase, 3.2.1.25
Laurate	Triacylglycerol lipase, 3.1.1.3
Palmitate	Triacylglycerol lipase, 3.1.1.3
Stearate	Triacylglycerol lipase, 3.1.1.3
Phosphate	Alkaline phosphatase, 3.1.3.1
Phosphate	Acid phosphatase, 3.1.3.2
Phosphocholine	Phospholipase C, 3.1.4.3
Sulfate	Arylsulfatase, 3.1.6.1
Thiobutyrate	Cholinesterase, 3.1.1.8
β -D-Xylopyranoside	β -D-Xylosidase, 3.2.1.37

ester, NY 14650) by recrystallizing from water twice, from dichloromethane once, and from water once again. The resulting four-times crystallized 4NP (Sample A in Tables 4 and 5) was then dried for several days in a desiccator at 50 °C under reduced pressure.

Sublimation. This process is performed as described (26) within an all-glass apparatus at a reduced pressure of about 100 N/m² (1 mmHg). Before attaching the condenser to the sample container, a quantity of 4NP, dissolved in a small volume of warm methanol, is put into the container. The sol-

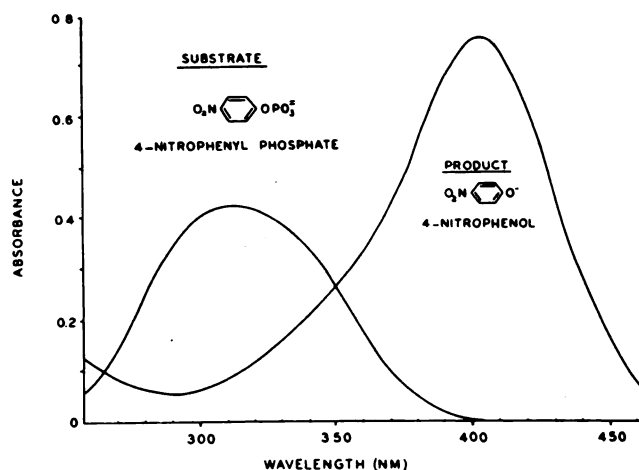


Fig. 1. Spectral absorption curves of 4-nitrophenol and 4-nitrophenyl phosphate in alkaline solution

vent is then allowed to evaporate so that a thin crystalline layer of 4NP is left adhering on the bottom and lower wall of the container to a height that is below the surface of the condenser. The apparatus is then connected to the source of reduced pressure. When under reduced pressure, the outer container is immersed in a water bath to the level of the 4NP crystals. Warm tap water is run through the condenser until the temperature of the water bath reaches about 60 °C, then cold tap water is used. Crystalline 4NP sublimes onto the cold surface as the bath is heated further. Heating is continued until most of the 4NP has sublimed.

Methods of Analysis and Results

Titration with alkali. We titrated samples of 4NP potentiometrically with a barium hydroxide solution that has been standardized against potassium acid phthalate (NBS-SRM no. 84h), which previously was finely powdered in an agate mortar and dried over magnesium perchlorate for 24 h. The barium hydroxide was ACS Reagent Grade, and the concentration of the solution, which had been prepared with boiled distilled water and protected from CO₂, was approximately 50 mmol/L. About 1 g of the phthalate or about 0.7 g of 4NP was weighed on a 50-mm watch glass in an analytical balance to the nearest 0.1 mg for each titration.

Table 2. The Reported Molar Absorptivity of 4-Nitrophenol

ϵ , L mol ⁻¹ cm ⁻¹	Wave- length, nm	Temper- ature, °C	Solution		pH	Analytical instrument (company, model)	Reference no.
			Type	Concn, mmol/L			
19 200	402.5	37	NaOH	1000	>11	Beckman DU	3
18 330	400	25	NaOH	10	>11	Unicam Uvispec	4
18 750	400	30	Tris	200	>8	Beckman DU	5
18 320	400	25	NaOH	1000	>11	Cary 14 PM	6
(18 380 at 402 mm)							
18 530	405	37	NaOH	75	>11	Eppendorf (Hg line photometer)	7
(18 350 to 18 810)							
18 400	401	25	NaOH	10	>11	Beckman DU Perkin-Elmer 202	8
18 380	401	25	NaOH		>11	Cary 16	9
18 300 to 19 200	401 to 404	25 to 37	^a	^a	^a	Cary 16	10
18 600 ^b	401	25	diethanol- amine	1000	9.8	Eppendorf 1101 Eppendorf 1101 M	11
(18 000 to 20 000)							
18 380 ± 90	401	25	NaOH	10	>11	Cary 219	this report

^a See Figure 2, this paper. ^b This report gave values in four solutions: (a) diethanolamine alone (as listed); (b) same as (a), except 1 mmol of magnesium added per liter; (c) same as (b), except 10 mmol of 4NPP added per liter; and (d) same as (c), except 5 g of serum albumin added per liter.

Table 3. Characterization of Alkaline Phosphatase Determinations in Which 4NPP Is Used as Substrate

Method (ref.)	Bowers & McComb (8)	German Committee (13)	Scandinavian Committee (14)	Selected Methods (AACC) (15)	Munan et al. (SMA 12/80) (17)	Van Belle (16)	Fleisher et al. (21)	French Committee (19)	McComb et al. (2)
Reagents									
4NPP, mmol/L	4	10	10	15	5	5	14	16	16
Buffer type, ^a , mmol/L	X,750	Y,1000	Y,900	X,800	X,650	Z,100	Y,1000	Y,900	X,1000
Final pH	10.15	9.8	9.8	10.3	10.2	10.2	10.15	10.5	10.5
Mg ²⁺ , mmol/L	0.1	0.5	0.5	0.1			0.1	1	(4)→1
Conditions									
Reaction temp., °C	30	25	37	30	37	30	30	30	29.77
Sample size, μ L	100	20	10	100	—	50	10	100	50
Vol ratio (sample/total)	1/30	1/101	1/111	1/30	—	1/51	1/27.1	1/30	1/61
Molar absorptivity of 4NP^b									
Wavelength (nm),	402	405	405	402	^c	405	402	402	402
L mol ⁻¹ cm ⁻¹	18 750	18 500	19 000 ^d	18 800	19 150 ^d	18 500 ^d	18 800 ^d	18 800	19 050
Adult upper reference limit for alkaline phosphatase, U/L									
	80	170	375 ^d	90	130	100	220 ^d	100 ^d	100

^a X, 2-amino-2-methyl-1-propanol; Y, diethanolamine; Z, ethylaminoethanol. ^b Under the assay conditions listed above for each method, the molar absorptivity in buffers is usually higher than in NaOH (see Figure 2). ^c Wavelength not specified. ^d Values estimated by one of us (GNB, Jr.).

In performing the titrations, we placed 50 mL of distilled water (previously boiled to remove CO₂) in a beaker, stirred with a magnetic stirrer, and inserted a pH electrode into the beaker. The potassium acid phthalate or the 4NP was washed from the watch glass into the beaker and the titration was begun. The solid material dissolved as the titration progressed. The end-point of the titration for potassium acid phthalate was pH 8.9 and for the 4NP was 9.7.⁵ Table 4 gives results of the titrations for several samples of 4NP.

Differential scanning calorimetry. This was done with a Model DSC-1B instrument (Perkin-Elmer Corp., Norwalk, CT 06856). Sample size was approximately 2 to 3 mg, weighed to the nearest microgram. The heating rate was 0.625 °C/min. High-purity indium metal was used as a standard, both for the heat of fusion and for determining the melting slope of a pure compound.

We used a computer program to reduce the data and to plot absolute temperature against the fraction of the material melted, T vs $1/f$. The slope of the line is equal to the freezing point depression. From that value and the heat of fusion, which is also obtained from the data, the mole-percent impurity is calculated (27). Table 4 lists results obtained for various samples of 4NP.

Water content. We determined the water content of a few samples of 4NP by Karl Fischer titration with a Metrohm Karl Fischer-Automat E547 titrator (distributed by Brinkmann Instruments Inc., Westbury, NY 11590). Sample masses of 0.5 to 1.0 g were used, each weighed to the nearest 0.1 mg. The detection limit for water at the reagent strength used was about 10 μ g. These results are also presented in Table 4.

Liquid chromatography. Several liquid-chromatographic separations were tested in an attempt to detect and identify impurities in samples of 4NP. A method similar to that described by Culbreth et al. (28) was the most useful. Conditions were: μ Bondapak C-18 column (Waters Associates, Inc., Milford, MA 01757); flow rate 2.0 mL/min; detection at 254

nm, 0.1 A full scale; solvent, Waters PIC-A reagent in water/methanol, 60/40 by volume; and 20- μ L injections of a 2 g/L solution. Separations of 2-, 3-, and 4-nitrophenol reference solutions were good. Injections of solutions containing 40 μ g of 4NP gave peaks with amplitudes that were about 10 times full scale. We detected no peaks corresponding to the other nitrophenols in any of the samples studied. We believe the limit of detection is about 0.1% for the isomeric nitrophenols under these conditions.

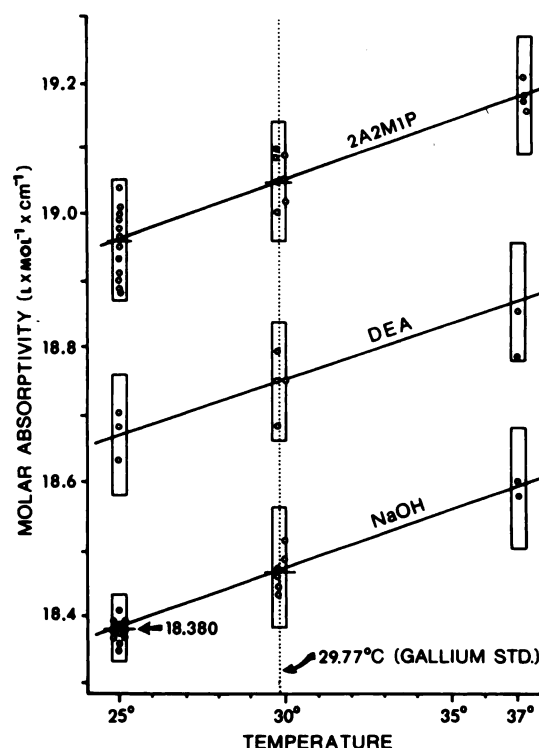


Fig. 2. Effect of temperature and reaction media on the molar absorptivity of 4-nitrophenol

NaOH concn, 10 mmol/L; diethanolamine (DEA) 1 mol/L, pH 10 at 25 °C; 2-amino-2-methyl-1-propanol (2A2MIP) 1 mol/L, pH 10.5 at 29.77 °C. The spectrophotometric measurements at 25.0, 29.77 and 37.0 °C were made at 401, 402.5, and 404 nm, respectively. y-axis, $\epsilon \times 10^{-3}$

⁵Based on pK_a of 5.1 for potassium acid phthalate, the pH at its equivalence point was calculated to be 8.9, with a water blank of 8 μ L. Based on a pK_a of 6.7 for 4NP, the pH at the equivalence point was calculated to be 9.7, with a water blank of 50 μ L. The final volume per titration was about 100 mL.

Table 4. Water Content and Estimates of Purity of Different Preparations of 4NP

Sample	Description of the materials	Water content (mass %)	Alkali titration (mass %)	Differential scanning calorimetry (mol %)
A	Procedure A—4X recrystallized from Eastman P192 (Hartford)	—	—	99.97
A ₁	Same as A but orange from exposure to light after stored in room air 1 yr	—	99.88	99.87
B	Procedure B, sublimed from Baker (99 %)-(NBS)	0.01	99.95	—
B ₁	Same as B but stored in dark 1 yr in original container (not discolored)	—	—	99.90
B ₂	Same as B but orange from exposure to light over 1 yr in clear closed container	—	—	99.90
C	Sigma "Spectrophotometer Standard" 104-8, Lot No. 103C-5610	0.25	99.56	(99.5 ± 0.2)*
1	E. Merck No. 6798, Lot 9704164	—	—	99.88
2	Eastman "99 %" No. 192, "new" bottle (NBS)	—	99.77	—
3	Eastman "99 %" No. 192, "old" bottle (NBS)	—	99.62	99.85
4	Sample 3 recrystallized from water (NBS)	—	99.95	99.95
5	Sample 4 after storage for one year (NBS)	0.15	99.90	—
6	Recrystallized 2X from water in 1970, unknown source (Hartford)	—	—	99.92
7	Original purity 70%; 2X recrystallized from water (Hartford)	—	—	99.94

These analyses were made at NBS between 1977 and 1979, except for the one marked *, which was determined in 1974 by courtesy of Dr. Bruce Cassel, Perkin-Elmer Corp., Norwalk, CT 06856. Additional data on Samples A, B, and C are given in Table 5.

Table 5. Molar Absorptivity Values Found for High-Purity 4NP

Cary 219 (Hartford)	Sample A ^a (4X recrystallized)	Sample B (sublimed)	Sample C ^a (Sigma 104-8)
December, 1978	18 355	18 404	18 374
January, 1979	18 369	18 364	18 371
January, 1979	18 358	18 355	18 382
	$\bar{x} = 18\,361$	$\bar{x} = 18\,374$	$\bar{x} = 18\,376$
NBS high-accuracy instrument ^b			
January, 1979	18 399	18 385	18 378

^a Dried in a desiccator at reduced pressure and 50 °C before assay.

^b Measurements courtesy of R. Burke and J. R. Baldwin, Center for Analytical Chemistry, NBS.

Spectrophotometry. The spectrophotometric measurements were made at Hartford Hospital with a Cary Model 219 spectrophotometer (Varian Associates, Palo Alto, CA 94303) and at NBS with the state-of-the-art spectrophotometer (29). At Hartford, air was used in the reference beam and solutions were measured in a single quartz cell (SRM No. 932) whose pathlength was certified by NBS to 5 parts in 100 000 (30). The absorbance of the blank (solution containing all reactants except 4NP) was measured in the same cell and was subtracted from that for the corresponding solution containing 4NP. The natural bandwidth of 4NP is about 66 nm (24); the maximum spectral bandwidth we used was 1.7 nm at 401 nm and 2.2 nm at 440 nm. Inaccuracy of wavelength settings did not exceed 0.2 nm, either at the 360.9-nm peak absorbance of a holmium oxide glass filter (31) or at the 365.02-nm and 404.66-nm emission lines of the mercury lamp provided as a calibration accessory with the Cary instrument. We checked photometric accuracy of the Cary 219 by use of NBS/SRM 930b glass filters (32) and also by comparison with acid dichromate, as described elsewhere (33, 34). Further to assure the comparability of the spectrophotometric measurements on 4NP made in the Hartford and NBS laboratories, the set of SRM 930b glass filters used in Hartford was recalibrated at NBS. All absorbance values fell within the ±0.002 certified tolerance limits assigned three years earlier ($A_{440\text{nm}} = 1.042$ for No. 347-1; $A_{440\text{nm}} = 0.714$ for No. 347-2; and $A_{440\text{nm}} = 0.572$ for No. 347-3). Likewise, at the time of making measurements on Samples A, B, and C of Table 5, all absorbance

Table 6. Specifications for High-Purity 4-Nitrophenol (Formula weight = 139.1)

Appearance	colorless to slightly yellow crystals
Odor	none
Melting point	113–114 °C
Titration with alkali	99.9 ± 0.1 g/100g
Water content	<0.10 g/100g
Liquid chromatography	no impurities detectable
Differential scanning calorimetry	99.9 ± 0.1 mol/100mol
Spectrophotometry	18 380 ± 90 L·mol ⁻¹ ·cm ⁻¹

values on these SRM 930b glass filters obtained with the Cary 219 also lay within these certified limits.

For the spectrophotometric readings on 4NP given in Table 5, about 139 mg (1 mmol) of high-purity 4NP (i.e., Samples A, B, and C), weighed to the nearest 0.01 mg, was quantitatively transferred to a preweighed 1-L volumetric flask. After dissolving the 4NP in reagent-grade water (conductivity < 1 × 10⁻⁶ Ω⁻¹ cm⁻¹), filling to the mark and mixing, we determined the mass of the solution by weighing the flask and contents, and calculated the weight ratio of 4NP in the solution.

Approximately 35 mL of this solution was dispensed into a 1-L Class A volumetric flask from a plastic syringe (with needle attached). The mass of transferred 4NP solution was determined by weighing the syringe when filled and after delivery. (The calibration of each Class A volumetric flask used was checked and was well within the specifications of 3 parts in 100 000.) Reagent-grade water and 10 mL of 1 mol/L NaOH were added and, after temperature-equilibration in a water bath set at 25.0 °C, the flask was filled to the mark. Complete mixing was ensured by inverting each flask at least 30 times. The concentration of 4NP was calculated in moles per liter.

Immediately after recording each spectrophotometric reading, we measured the temperature of the solution within the quartz cuvette to the nearest 0.1 °C with a gallium-calibrated (NBS SRM no. 1968) electronic thermometer equipped

with a rapidly responding microthermistor (Model 45CU; Yellow Springs Instrument Co., Oberlin, OH 45387) (35). The absorbance at 25.0 °C was determined by repeating the spectrophotometric and temperature measurements on the same solution in the cuvette and plotting the absorbance temperature. The average absorbance at 25.0 °C was obtained from plots of three or more fresh samples of the final solutions of the high-purity 4NP samples under study, and was used to calculate the molar absorptivities in Table 5.

Discussion

Because commercially supplied 4NP preparations are not uniformly of high purity, the user is advised to assay the 4NP to determine its relative purity. Our purification and characterization studies with many lots of 4NP from numerous sources have provided the basis for developing specifications for an acceptable 4NP reference material. Fortunately, acceptably pure material is readily available from a number of commercial suppliers. High-purity 4NP should be colorless to only slightly yellow, have no odor, and melt at 113–114 °C. It is also relatively easy to prepare material of acceptable purity by either the multiple crystallization or the sublimation techniques described in this paper.

As shown in Table 4, water may contribute measurably to the amount of impurity. The water-content analyses by Karl Fischer titrations agreed with the impurity estimations given by alkali titration. Note that Sample C in Table 4 had measurable water, but after being dried at reduced pressure and 50 °C for 24 h it gave molar absorptivity values in excellent agreement (Table 5) with Samples A and B. High-purity 4NP should have a water content of <0.10 g per 100 g.

The values in Table 4 for 4NP purity by titration with alkali are reasonably consistent with those by differential scanning calorimetry. Note that one method gives purity in terms of grams per 100 grams ("mass percent") and the other as moles per 100 moles ("mole percent"). We estimate the imprecision of these measurements to be about 0.05% (CV). Because the two methods give reasonably consistent results, we estimate that the magnitude of their systematic errors are small although unknown. The titrimetry estimate includes the assumption that there are no acid impurities in the 4NP. With the calorimetry estimate, one assumes that all impurity molecules are detected, which in turn depends on all impurities being insoluble in the solid phase but soluble in the melt. High-purity 4NP should give alkali titration measurements of 99.9 ± 0.1 mass percent and differential scanning calorimetry values of 99.9 ± 0.1 mole percent.

Despite repeated attempts to analyze for them by liquid chromatography, we did not encounter 2- and 3-nitrophenol as impurities at an estimated detection limit of about 0.1%. On the other hand, the colored substances of unknown structure that are largely removed by recrystallization or sublimation may persist or may reappear to some small extent in the purified samples for which measurements are shown in Table 4. The orange color that appears after long exposure to light does not seem to cause identifiable changes (compare A_1 with A and B_2 with B_1 in Table 4).

As shown in Figure 2, changes in temperature result in changes in the absorbance of 4NP in alkaline solution. Despite the special attention we gave to the control, careful measurement, and correction of all solution temperatures to 25 °C during spectrophotometric readings, we judge that errors related to temperature might account for a variability of approximately 0.1% in the values listed in Table 5. The error associated with pathlength should be <0.1% because the SRM 932 reference quartz cuvettes have been certified by NBS to 5 parts in 100 000 (30); the internal pathlength of our cuvette was given as averaging 9.999 mm. We estimate the residual impurities in high-purity 4NP dried under reduced pressure

(such as Samples A, B, or C listed in Table 4) to be < 2 mg/g.

Thus, the accumulated uncertainties from the determination of absorbance (A), the preparation of known concentrations of 4NP (c), the pathlength (b), and the temperature permit calculation of the molar absorptivity of 4NP in 10 mmol/L aqueous NaOH at 401 nm at 25 °C by the formula:

$$\epsilon = [A/(bc)] \text{ (L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}\text{)}$$

A mean value of $18\,378.8$ (SD 12.6) $\text{L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ was obtained for six determinations (see Table 5—three mean values from Hartford and three single values from NBS), giving a std. error of the mean of $5.1 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. The random error was taken as $3 \text{ SEM} \approx 15 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ and the systematic error was estimated to be $\approx 75 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ by use of the propagation-of-errors formula similar to that in our prior publication (34). No corrections were made for multiple reflections from cuvette surfaces, beam convergence, or air buoyancy, because such factors would also be present to a like extent for others using high-resolution spectrophotometers as well as for most others making pragmatic determinations of enzyme catalytic concentrations.

Table 6 summarizes our recommendations for the specifications of high-purity 4NP suitable as a spectrophotometric reference material. Our best estimate of the molar absorptivity of 4NP and its overall uncertainty under the conditions mentioned above is $18\,380 \pm 90 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$.

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Ed Note: The authors of this paper wish to call attention to a paper bearing on this subject that came to their attention after their paper was in press: Jung, K., and Köhler, A., On the influence of reaction conditions in activity determinations of alkaline phosphatase on the molar absorptivity of 4-nitrophenol. *Clin. Chem. Acta* **101**, 1-4 (1980).