control was isolated directly as the hydrazone derivative. In an experiment similar to that described in the last section, the unoxidized L-leucine remaining on the column after washing with water was eluted with 2 *N* HCl. The eluate was evaporated to dryness several times in a rotating evaporator. The residue was dissolved in water, cleared by centrifugation and neutralized with 1.6*N* NaOH. Five volumes of alcohol were added and the solution cooled and the crystalline L-leucine centrifuged off. The leucine was then recrystallized in the same manner and finally taken to dryness over P_2O_5.

The isolated compounds were then analyzed for deuterium content*. The results are shown in Table I.

TABLE I

Compound recovered	*moles deuterium/mole*
L-leucine	~0
Phenyl hydrazone (enzymic reaction)	0.12
Phenyl hydrazone (non-enzymic reaction)	0.15

It is unlikely that any significant exchange occurred on the column since the α-keto *iso*caproic acid is not bound to the resin and the medium in which the acid is eluted from the column is at least 50% D_2O.

Thus it is quite apparent that the deuterium which is incorporated into the α-keto *iso*caproate is a result of enolization only and that no other deuterium from the medium is incorporated. Furthermore no significant reversal occurred under the reaction conditions since no excess deuterium is incorporated into the L-leucine. The oxidation of amino acids by L-amino acid oxidase must therefore proceed through the imino form of the amino acid according to mechanism (1) and an α–β unsaturated intermediate as in mechanism (2) is not formed.

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Interference by reduced pyridine nucleotides in the diazotization of nitrite

Nitrate and nitrite reductases are usually assayed in plant extracts by determining the formation or removal of NO_2^-, using either di- or triphosphopyridine nucleotide (DPNH or TPNH) as an electron donor[1,2,3,4]. Sulphanilamide is added to form a diazo compound with NO_2^-, which is then coupled to N-(1-naphthyl)-ethyldiamine hydrochloride to form the red azodye[5]. It has been observed that a small non-enzymic disappearance of nitrite occurs when TPNH and nitrite are allowed to react for a few minutes with the acid sulphanilamide reagent prior to coupling it to the naphthyl reagent[6]. This has been attributed to the deamination of the amino group of the adenine in TPNH.

During the course of our enzymic studies on nitrate assimilation in plants, we have confirmed that TPNH or DPNH interfere with the nitrite test. When determining the appearance of NO_2^- from NO_3^- in the nitrate reductase assay the effect of the TPNH or DPNH is negligible because NO_2^- is being formed. There is, however, considerable interference in the nitrite reductase method, because NO_2^- is being removed and the DPNH required for maximal activity of the enzyme results in more than 75% non-enzymic disappearance of NO_2^-, as shown in Table I.

TABLE I

ASSAY OF NITRITE REDUCTASE FROM *Neurospora crassa*

(mμmoles NO_2 disappearing/10 min incubation period)

Experiment	*1*	*2*	*3*
Complete reaction mixture	15	14	16
Reaction mixture less enzyme	9.4	11.1	11.1

Complete reaction mixture: 0.15 ml 0.1*M* pyrophosphate (pH 7.5); 0.2 ml 10^{-4}*M* $NaNO_2$; 0.05 ml boiled pig heart; 0.1 ml 10^{-3}*M* DPNH and 0.1 ml enzyme (1.56 mg protein/ml). After 10 min incubation, 0.5 ml 1% w/v sulphanilamide in *N* HCl and 1 ml 0.01% w/v N-(1-naphthyl)-ethylenediamine hydrochloride were added and the volume made to 5 ml with distilled water. The solutions were assayed in a Spekker absorptiometer in 0.5 cm cells, with green filters.

TABLE II

INHIBITION OF THE NO_2 TEST BY DPNH

Expt.	Optical density		Inhibition by DPNH (%)
	20 mμmoles NO_2	20 mμmoles NO_2 + 0.1 μmole DPNH	
1	0.079	0.027	68
2	0.077	0.025	69
3	0.080	0.026	69

Assay for NO_2 as in Table I.

In these experiments the naphthyl reagent was added immediately after the sulphanilamide but delay in adding the latter made no difference to the results.

The data in Table II show the effect of DPNH in inhibiting the nitrite test.

These results cannot be interpreted to mean that a chemical reduction of NO_2^- by DPNH has occurred because there was no oxidation of DPNH by NO_2^-, in the absence of the enzyme, as illustrated in Fig. 1.

EVANS[7] has shown that, under acid conditions, the presence of DPNH did not result in an appreciable disappearance of NO_2^- unless the ratio of DPNH to NO_2^- was 5:1. The results of our experiments, however, demonstrate that the interference is considerable over a wide range of DPNH/NO_2^- or TPNH/NO_2^- ratio, *viz.* 2.0 to 10, as shown in Table III. At an equivalent ratio, the inhibition by DPNH is more severe than that of TPNH.

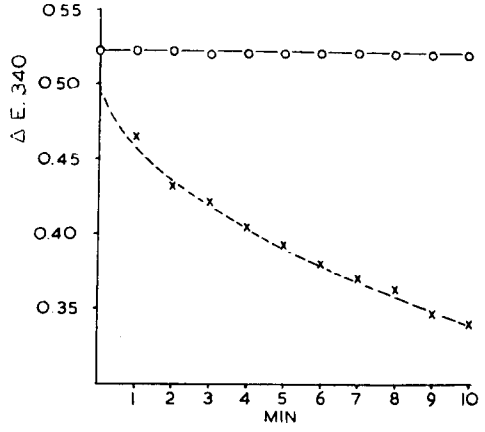

Fig. 1. Effect of NO_2^- on the oxidation of DPNH in the presence and absence of nitrite reductase. O——O, 20 mμmoles NO_2 and 0.1 μmole DPNH in 0.1 *M* pyrophosphate pH 7.5; ×——×, as above plus 0.1 ml nitrite reductase (1.5 mg protein/ml) from *Neurospora*. Ordinate: log I_0/I at 340 mμ.

TABLE III

EFFECT OF VARIOUS MOLE RATIOS OF DPNH/NO_2 AND TPNH/NO_2 ON THE NITRITE TEST

(Values found as % of NO_2 added)

DPNH/NO_2	*0*	*2.5*	*5*	*7.5*	*10*
Expt. 1	100	50	46	30	25
Expt. 2	100	50	44	31	24
TPNH/NO_2	*0*	*2*	*4.1*	*6.2*	*8.3*
Expt. 3	100	80	75	57	50
Expt. 4	100	77	74	55	48

TABLE IV

EFFECT OF THE ORDER OF ADDING REAGENTS ON THE DPNH INHIBITION OF THE NITRITE TEST

(Values found as % of NO_2 added)

Order of addition of reagents	*1. NO_2 2. Sulphanilamide 3. Naphthyl reagent*	*1. NO_2 2. DPNH 3. Sulphanilamide 4. Naphthyl reagent*	*1. NO_2 2. Sulphanilamide 3. DPNH 4. Naphthyl reagent*	*1. NO_2 2. Naphthyl reagent 3. DPNH 4. Sulphanilamide*
Expt. 1	100	34	100	60
Expt. 2	100	32	100	60

Assay mixture: 20 mμmoles NO_2 and 0.1 μmole DPNH; NO_2 determined as in Table I.

The mode of the DPNH interaction with nitrite was studied further by varying the order in which the reagents were added to the reaction mixture. The results are shown in Table IV.

It is clear that when sulphanilamide reacted first with NO_2^- to form the diazonium salt the subsequent addition of DPNH did not interfere with the azodye formation. Thus it appears that DPNH and TPNH compete with NO_2^- for the same site on the diazo compound. It is of interest that neither DPN nor TPN inhibit the diazotization process.

To overcome this serious interference with the nitrite reductase assay we propose the following modification in which residual DPNH and TPNH are removed by a barium acetate-alcohol treatment. At the end of the incubation period add 0.1 ml *M* Ba acetate and 2.5 ml 95% v/v ethanol, in the cold, to the reaction mixture, agitate well and centrifuge at 0° C. Nitrite is determined in the supernatant, in the usual way. The results obtained by this method are reproducible and devoid of interference.

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Correction of rotatory dispersion characteristics of adenosine triphosphate and related compounds

In a recent publication[1] the influence of pH on the value of λ_c and K_m of adenine, adenosine, AMP, ADP and ATP was presented in table-form. Because of a constant arithmetic error in the calculations, all values of slope (K_m), standard deviation of slope and molecular rotation are too large by a factor of 2. The intercept (λ_c) and the standard deviation of the intercept are not affected. It should be noted that this error in no way affects the ideas or interpretations presented in the publication.

A corrected table is given below.