As shown in Fig. 5, all isotherms are of the Langmuir type. Each curve starts from the origin and the amount adsorbed reaches a saturation value at or close to the CMC in solution. The areas per molecule calculated from the saturation adsorption were considerably larger than the values for the air–solution interface and were also larger than the dimensions of the hydrocarbon part of the molecule given by a molecular model. With an increasing polyoxyethylene chain length, the value of saturation adsorption decreases, but this trend is not so evident as compared with sulfathiazole.

Figure 6 presents the sedimentation volume of naphthalene in the presence of polyoxyethylated nonyl-phenols. At very low concentrations, naphthalene was only slightly wet by water and some portions of it floated, so measurement of the sedimentation volume was difficult. The sedimentation volume rapidly decreased with an increase in concentration and a highly deflocculated state was reached. The deflocculating action of the surfactants was not so influenced by the polyoxyethylene chain length.

It is likely, therefore, that the hydrocarbon chains of the surfactant molecules adhere to the surface of naphthalene and are lying flat, while the polyoxyethylene chains are directed toward solution.

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GLC Determination of Nitroglycerin and Isosorbide Dinitrate in Human Plasma

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Abstract □ A procedure is described for the identification and quantitative determination of nitroglycerin and isosorbide dinitrate in plasma. The nitrates are extracted from plasma with ethyl acetate and measured by GLC with electron-capture detection. Quantitation is done using an internal standard. The lower limit of sensitivity for both substances is around 0.5 ng./ml. Results obtained with this method in humans are given.

Keyphrases □ Nitroglycerin—identification, GLC–electron-capture analysis, human plasma □ Isosorbide dinitrate—identification, GLC–electron-capture analysis, human plasma □ GLC–electron-capture detection—identification, analysis, nitroglycerin and isosorbide dinitrate in human plasma

Knowledge of the metabolic fate of the antianginal organic nitrates in man is hampered by a lack of sensitive analytical methods; attempts to measure plasma levels in man by colorimetric methods after administration of these nitrates were clearly unsuccessful (1, 2). The use of labeled substances has allowed the detection of small amounts of pentaerythritol tetranitrate in man (3, 4); for nitroglycerin and isosorbide dinitrate, the labeled compounds have been used in animals only (5–8).

The present authors previously described a GC method for the separation and identification of different organic nitrates in the nanogram range, using electron-capture detection (9). Only incomplete data about electron-capture detection for assay of organic nitrates in biological material have been published (10).

This report describes the use of GLC for the identification and quantitation of nitroglycerin and isosorbide dinitrate in plasma after administration of therapeutic doses in man.

EXPERIMENTAL

Reagents—Benzene[1], trimethylchlorosilane[2], and activated charcoal[3] filters were used. Ethyl acetate[1] was shaken three times with 10% ferrous sulfate and two times with water distilled in glass, stored over calcium chloride (95% minimum)[2], and distilled immediately before use. Anhydrous sodium sulfate[2] was washed with ethyl acetate and dried at 100° for several hours. Nitroglycerin was available as a 1% solution in ethanol[2], and isosorbide

[1] RS per pesticidi, Carlo Erba, Italy.
[2] Merck, Germany.
[3] Norite, Selecta n° 508, ϕ 4 cm., Schleicher & Schüll, Dassel, Germany.

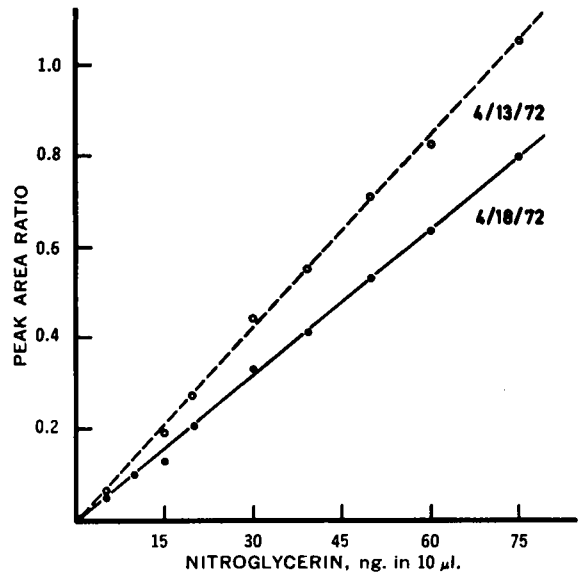


Figure 1—*Standard curves using plasma samples of 5 ml. to which are added 15 ng. of isosorbide dinitrate and varying amounts of nitroglycerin. From the extract in 10 μl. of benzene, 1.6 μl. is injected in the gas chromatograph with electron-capture detection. The two curves shown were obtained on 2 different days.*

dinitrate was available as a powder[4]; isoidide dinitrate was synthesized as described previously (11).

Glassware—All glassware was silanized by soaking for 1 hr. in 5% trimethylchlorosilane in benzene; rinsed with benzene, methanol, and water distilled in glass; and dried.

Apparatus—A gas chromatograph[5] with a tritium electron-capture detector (150 mc.) was used. Glass columns, 1.83-m. × 2-mm. i.d., were packed with 3.5% of QF-1[6] on 60–80-mesh Gas Chrom Q[7]. The injection port temperature was 160°, the column temperature was 120° for nitroglycerin determinations and 117° for isosorbide dinitrate determinations, and the detector block temperature was 180°. The nitrogen carrier gas flow was 30 ml./min. Pulse voltage was 25 v., and the electrometer range was 1×10^{-9}.

Extraction Procedure—Five milliliters of plasma and 5 ml. of ethyl acetate were shaken for 5 min. in glass-stoppered tubes; after centrifugation for 10 min. at 4000 r.p.m., the organic phase was transferred over an activated charcoal filter to a conical tube by means of a Pasteur pipet, avoiding the lipoprotein interface. The filter was rinsed before use with ethyl acetate and covered with sodium sulfate; afterward it was rinsed with 0.5 ml. of ethyl acetate. The organic phase was then evaporated under nitrogen to near dryness at room temperature. The plasma was extracted two more times. The yellow evaporation residue from the three extractions was dissolved in 0.5 ml. of ethyl acetate, filtered again through a filter, reevaporated to dryness, and immediately dissolved in 10 μl. benzene to prevent evaporation of nitroglycerin. The benzene solution (1.6 μl.) was injected into the gas chromatograph. The injection was done as soon as possible after the extraction procedure; in between the stoppered samples were stored at −18° to minimize evaporation of the benzene.

Quantitation—Quantitation of the amount of nitroglycerin or isosorbide dinitrate present in plasma was done by using an internal standard; this internal standard was isosorbide dinitrate when determining nitroglycerin and isoidide dinitrate when assaying isosorbide dinitrate. A known amount of the internal standard was added to the plasma samples before the extraction procedure. The peak areas of the nitrates on the chromatogram were calculated by multiplying peak height times width at half height; from the ratio of the peak areas of the product under study and the internal standard, the amount of product present in the samples can be calculated. A calibration factor was obtained by analyzing samples to which known amounts of both internal standard and test product were added. The presence of a wide front influences an early peak (*e.g.*, nitroglycerin) more than a late peak (*e.g.*, isosorbide dinitrate). Since the extraction solvent determines front width, the calibration samples are run through the whole extraction procedure used for the unknown samples (12).

[4] Cedona, The Netherlands.
[5] Packard Series 7400.
[6] Applied Sciences Laboratories.
[7] Supelco, Inc.

Table I—Efficacy of Extraction and Accuracy for the GLC Determination of Nitroglycerin and Isosorbide Dinitrate Added to Blank Plasma[a]

	Quantity[b], ng.	Mean Assay, ng.	*RSD*[c], %	*RE*[d], %
Extraction				
Nitroglycerin	25	24.3	±6.1	−2.8
Isosorbide dinitrate	15	14.5	±3.2	−2.9
Accuracy				
Nitroglycerin	10	10.2	±13.3	+2.2
Isosorbide dinitrate	5	4.9	±10.1	−2.0

[a] All determinations were done five times. [b] Added to 5 ml. of plasma. [c] Relative standard deviation. [d] Relative error.

RESULTS AND DISCUSSION

Retention Times—The relative retention time for nitroglycerin as compared to isosorbide dinitrate is 0.44; for isosorbide dinitrate as compared to isoidide dinitrate, it is 1.69. As described previously, nitrated metabolites of isosorbide dinitrate and of nitroglycerin have retention times different from those of the parent compounds (9); this is also true for the mononitrate of isoidide.

Extraction—From previous work (13) with TLC coupled with densitometry, it was known that microgram concentrations of nitroglycerin and isosorbide dinitrate can be extracted from plasma quantitatively. For concentrations in the nanogram range, the efficacy of extraction is given in Table I.

Activated charcoal filters are very helpful in working with biological material (14) but do not influence the impurities introduced by the use of the extraction solvent. Different brands of ethyl acetate were tried, as well as different purification methods, and the best results were obtained with the procedure described under

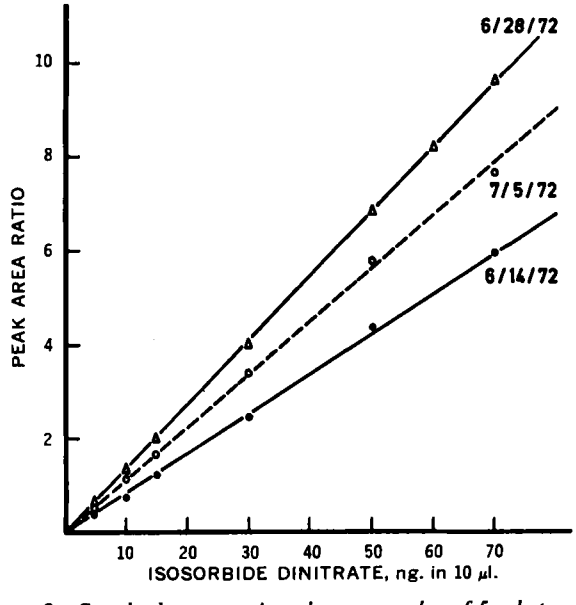


Figure 2—*Standard curves using plasma samples of 5 ml. to which are added 5 ng. of isoidide dinitrate and varying amounts of isosorbide dinitrate. From the extract in 10 μl. of benzene, 1.6 μl. is injected in the gas chromatograph with electron-capture detection. The three curves shown were obtained on 3 different days.*

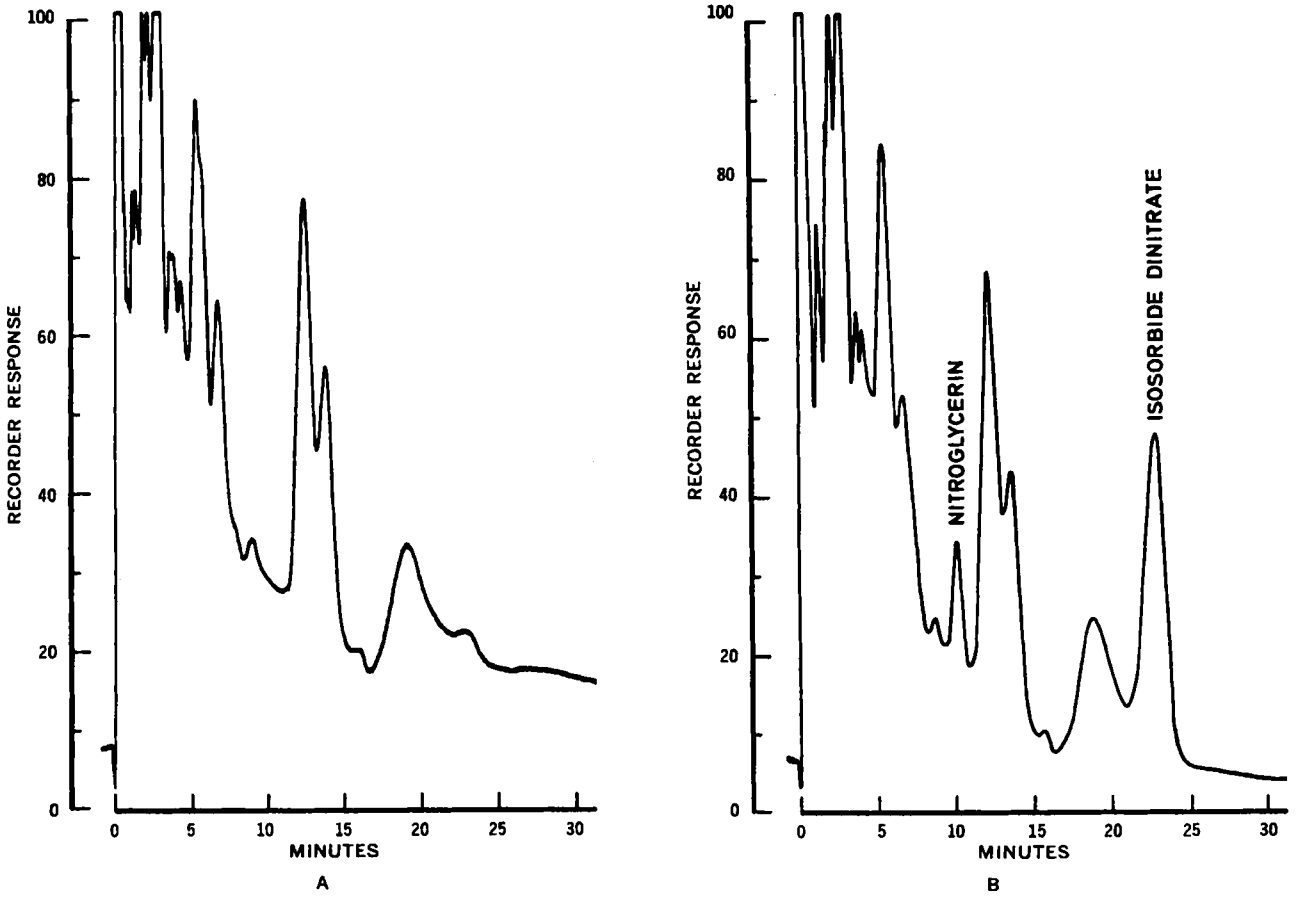


Figure 3—*Gas chromatograms of extracted human plasma. Key: A, extract from 5 ml. blank plasma; and B, isosorbide dinitrate (15 ng.) added to 5 ml. plasma of the same subject after sublingual administration of nitroglycerin, 800 mcg. In both cases the extraction was done with ethyl acetate, and 1.6 μl. of the extract in 10 μl. of benzene was injected.*

Experimental. Although the wide front and baseline irregularities still exist, none of these irregularities interferes with the peaks of the three nitrates. Some large peaks due to ethyl acetate impurities appear much later than the nitrates, so the chromatograms have to run for 90 min. before one can inject another sample.

Sensitivity—If 5 ml. of plasma is used for the extraction, the lower limit of detection is approximately 0.5 ng./ml. for nitroglycerin and somewhat lower for isosorbide dinitrate. As always with electron-capture detection, sensitivity varies from day to day; part of the variation is due to changes in front width and in baseline.

Quantitation—A prerequisite for the use of an internal standard for purposes of quantitation is a linear relationship between the ratio of the peak areas of internal standard and compound and the quantity of the compound. This is the case (Fig. 1) for control plasma samples of 5 ml. to which both nitroglycerin (5–75 ng.) and isosorbide dinitrate (15 ng.) were added. After extraction, 1.6 μl. of the 10-μl. benzene extract was injected into the gas chromatograph; the calibration line is linear and passes through the origin.

Table II—Plasma Levels (Nanograms per Milliliter) of Isosorbide Dinitrate at Different Times after Sublingual Administration of 1.25 mg. of This Substance[a] in Five Human Volunteers

Subject	Minutes[b] 4	6	15	30	50	80
M.B.	17.5	34.5	11.1	4.5	3.2	2.2
J.W.	3.1	14.4	5.2	3.8	2.7	1.0
M.D.	8.5	21.7	7.8	3.7	2.7	1.1
R.M.	15.8	11.5	2.6	1.2	1.9	0
M.V.	3.5	10.5	7.3	0.5	0.6	0.2

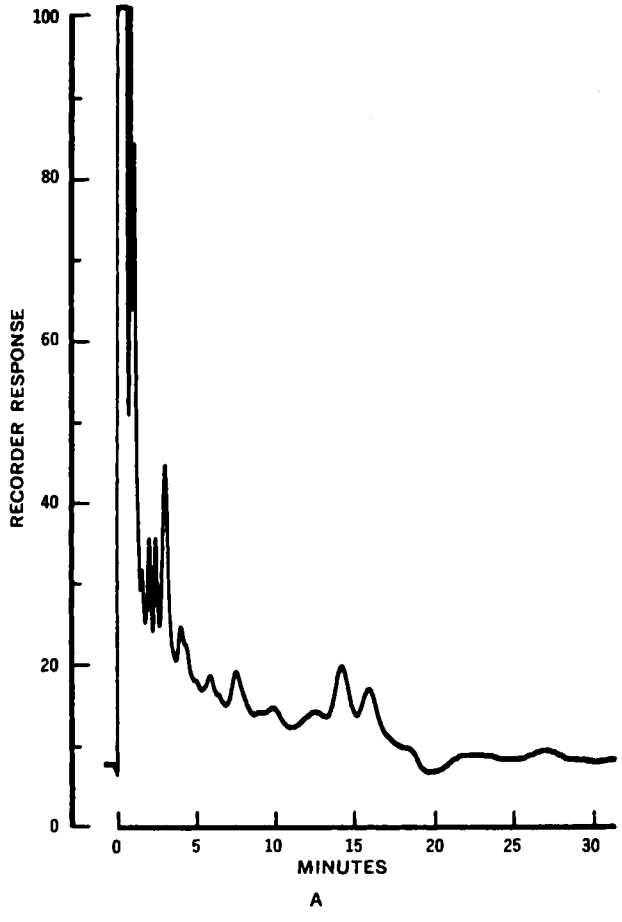
[a] A quarter of a commercially available tablet (Cedocard, 5 mg.) was put underneath the tongue. [b] Administration at $t = 0$ min.

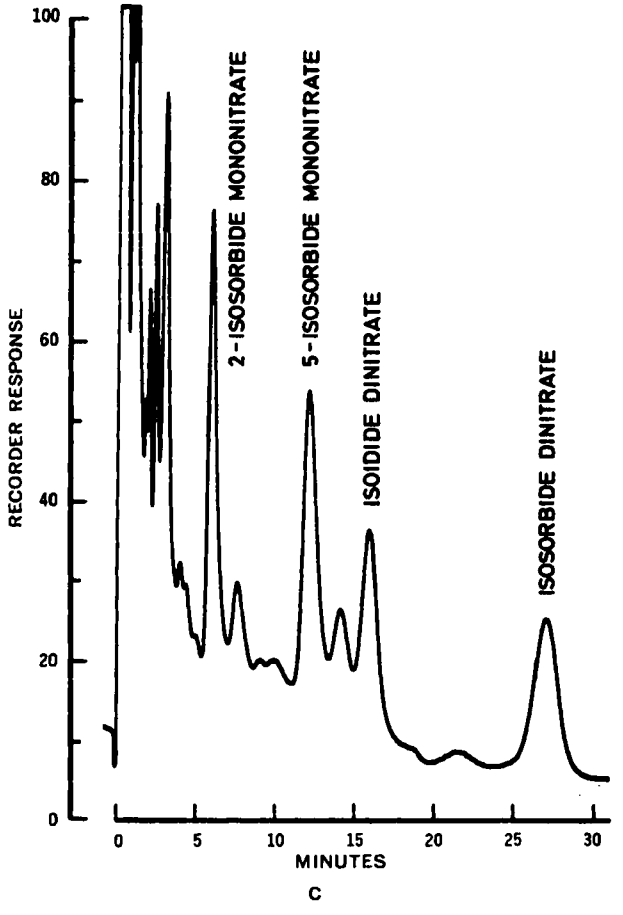
The slope of the curve is, however, different from day to day. Obviously, the length of the procedure precludes the preparation of a new calibration curve every day. Therefore, with each set of samples to be assayed for nitroglycerin, two calibration samples are run through the assay procedure: to each 5-ml. plasma sample, 15 ng. of isosorbide dinitrate and 25 or 50 ng. of nitroglycerin are added before the extraction procedure. From these two values, the slope of the calibration curve can be inferred.

For determination of isosorbide dinitrate, isoidide dinitrate is added as internal standard. Here, too, the relationship between ratio of peak areas and ratio of concentrations is linear, as ascertained by experiments in which 5 ng. of isoidide dinitrate and 5–70 ng. of isosorbide dinitrate were added to 5-ml. plasma samples (Fig. 2).

The accuracy of the method was assessed as follows. To each 5-ml. plasma sample, 10 ng. of nitroglycerin and 15 ng. of isosorbide dinitrate were added. After extraction and injection into the gas chromatograph, the nitroglycerin content was calculated from the respective peak areas, using a calibration factor obtained on the same day from a calibration sample containing 25 ng. of nitroglycerin and 15 ng. of isosorbide dinitrate for 5 ml. of plasma. Likewise, to plasma samples of 5 ml., 5 ng. of isosorbide dinitrate and 5 ng. of isoidide dinitrate were added. The isosorbide dinitrate content was calculated using a calibration sample containing 15 ng. of isosorbide dinitrate and 5 ng. of isoidide dinitrate for 5 ml. of plasma. The results of these accuracy tests are given in Table I.

As an illustration of the method, two chromatograms are shown in Fig. 3 from an experiment in which 800 mcg. of nitroglycerin was taken sublingually by a human volunteer. Figure 4 shows chromatograms from an experiment in which a volunteer was given 5 mg. isosorbide dinitrate orally; this figure also demonstrates the good separation of isosorbide dinitrate from its mononitrated metabolites.





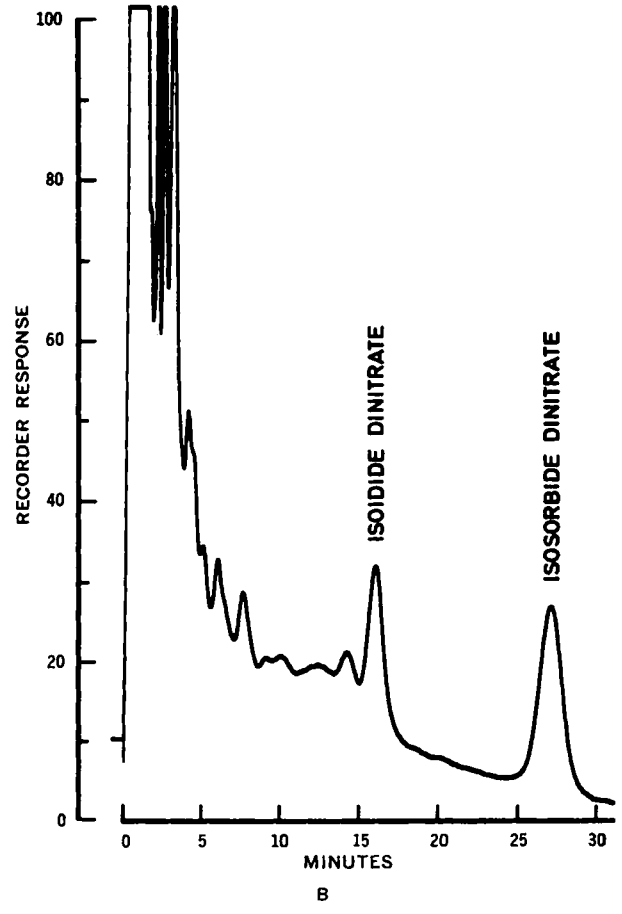


Figure 4—*Gas chromatograms of extracted human plasma. Key: A, extract from 5 ml. blank plasma; B, isosorbide dinitrate (15 ng.) and isoidide dinitrate (5 ng.) added to 5 ml. of blank plasma of the same subject; and C, isoidide dinitrate (5 ng.) added to 5 ml. of plasma of the same subject after oral administration of 5 mg. of isosorbide dinitrate. Plasma was extracted with ethyl acetate; and from the extract in 10 µl. of benzene, 1.6 µl. was injected into the chromatograph. Note the presence of mononitrated metabolites in the plasma after oral administration of isosorbide dinitrate.*

The method described here allows specific determination of small amounts of organic nitrates in plasma. Plasma levels of nitroglycerin, after sublingual administration of the substance in man using the method described here, have been published elsewhere (15). The results obtained with sublingual isosorbide dinitrate are given in Table II.

Use of the method described here for the study of orally given nitrates will hopefully lead to a better understanding of the problem of the long-term prophylaxis of angina pectoris by these products.

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Estimation of Changes Induced by Drugs in Cerebral Energy-Coupling Processes *In Situ* in the Dog

G. BENZI▲, E. ARRIGONI, L. MANZO, M. De BERNARDI, A. FERRARA, P. PANCERI, and F. BERTE

Abstract □ Drug action on the cerebral energy-yielding sequences was evaluated *in vivo via* the changes in the energy charge potential of the cerebral adenylate system and also the brain lactate and pyruvate systems. The two series of parameters were estimated before, during, and after hypoxemia in the hypovolemic, hypotensive beagle dog. The drugs were bemegride (6.4×10^{-4} *M*), nicergoline (1×10^{-4} *M*), and dipyridamole (2.5×10^{-4} *M*), which were perfused into the carotid artery at 0.5 ml./min. for 3 or 6 min. Bemegride reduces the energy charge, while dipyridamole and nicergoline increase the depressed cerebral energy charge potential. Nicergoline requires glucose and oxygen and is sensitive to malonate and cocaine.

Keyphrases □ Drugs—effects on cerebral energy-yielding processes, changes in charge potential of adenylate, lactate, and pyruvate systems using bemegride, nicergoline, and dipyridamole, beagle dogs □ Cerebral metabolism—effect of bemegride, nicergoline, and dipyridamole on energy-yielding processes, effect on adenylate, lactate, and pyruvate systems, beagle dogs □ Energy charge, cerebral—changes induced by bemegride, nicergoline, and dipyridamole before, during, and after hypoxemia in hypovolemic, hypotensive beagle dogs □ Metabolism, cerebral—drug-induced changes in energy-coupling processes, bemegride, nicergoline, and dipyridamole effects, beagle dogs □ Bemegride—effects on cerebral energy-coupling processes, beagle dogs □ Nicergoline—effects on cerebral energy-coupling processes, beagle dogs □ Dipyridamole—effects on cerebral energy-coupling processes, beagle dogs

Quantification *in vivo* of the action of drugs that can interfere in the cerebral energy-yielding sequences or that can modify cerebral reactions at metabolic branch points is a problem involving theoretical and methodological considerations. Since several experimental conditions such as anesthesia, hypothermia, or hypercapnia can produce large changes in the cerebral oxygen metabolic rate without disrupting the energy balance (1–3), changes in oxygen consumption cannot be used to define the action of a drug on the cerebral energy state. On the other hand, not all of the factors regulating the cerebral blood flow have been clarified, so flow changes cannot readily be used to evaluate drug action on energy states.

Furthermore, recent results have demonstrated that the phosphocreatine and lactate concentrations and the